2015

Therapeutic Drug Monitoring of Immunosuppresive

Mwlod A. Ghareeb
University of Rhode Island, maghareeb@yahoo.com

THERAPEUTIC DRUG MONITORING OF IMMUNOSUPPRESSIVE

BY

MWLOD A. GHAREEB

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
BIOMEDICAL AND PHARMACEUTICAL SCIENCES

UNIVERSITY OF RHODE ISLAND
2015

DOCTOR OF PHILOSOPHY DISSERTATION

OF

MWLOD A. GHAREEB

APPROVED:

Thesis Committee:

Major Professor Fatemeh Akhlaghi

Sara Rosenbaum

Liliana Gonzalez

Nasser H. Zawia

DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND
2015

ABSTRACT

The immunosuppressive agents used to prevent rejection of transplanted organs include cyclosporine (CsA), everolimus (EVE), mycophenolic acid (MPA), prednisolone (PLN), sirolimus (SIR) and tacrolimus (TAC). Because of the narrow therapeutic index and high inter- and intra-subject variability of these agents, therapeutic drug monitoring (TDM) is an integral part of immunosuppressive therapy following organ transplantation. The immunosuppressants incidence and severity of side effects correlate with the degree of exposure while under-dosed patients can be at a greater risk for allograft rejection. Currently, whole blood or plasma samples that are obtained via venipuncture are used for routine immunosuppressive monitoring. The limitations of venipuncture blood samples include (i) invasive nature associated with the sample collection and (ii) weak correlation with the drug concentration at the site of action. This thesis is consisted of the following sections written in a manuscript format.

Manuscript I provides a comprehensive review of literature published on alternative techniques that are proposed to overcome the limitation of venipuncture sampling. These methods include the use of non-conventional techniques, namely, drug monitoring in oral fluids or blood samples obtained from fingertip as well as drug concentration measurement in lymphocytes or transplanted tissue.

Drug concentration measurement in lymphocytes or transplanted tissue is primarily aimed at obtaining information on drug level at the site of action thus to facilitate prediction of clinical outcomes. However, these approaches are impractical in clinical

setting because of the invasive nature of sampling as well as complicated sample preparation procedures.

The objective of finger prick sampling is to mitigate the discomfort and difficulties associated with venipuncture, especially in pediatrics and frail patients. In this approach, the fingertip blood samples are either applied onto a filter paper (dried blood spots) or are processed as a liquid. It has been reported that fingertip sampling was preferred to venipuncture by both patients and healthcare providers. Nevertheless, the main disadvantages of venipuncture whole blood sampling, which is the poor correlation with concentration at the site of action**,** still exist.

Finally, oral fluid sampling is a promising non-invasive method of therapeutic monitoring of immunosuppressive agents. Advances in analytical techniques have enabled measuring drug concentration in minute amount of sample. Drug concentration in oral fluids represents the free fraction which should theoretically represent drug concentration at the site of action.

Few comprehensive studies investigated the use of oral fluids as a medium for therapeutic drug monitoring. Therefore, this dissertation is focused on the development of sensitive and robust liquid chromatography tandem mass spectrometry methods for quantification of the most commonly used immunosuppressant agents, tacrolimus and mycophenolic acid. The methods are then used to quantify these agents in oral fluids samples collected from kidney transplant recipients.

Manuscript II describes, in details, the development and validation of a liquid chromatography tandem mass spectrometry (LC-MS/MS) method for quantification of tacrolimus in oral fluids. This method was validated in accordance with the current

Food and Drug Administration (FDA) guideline. The Lower Limit of Quantification of this method is 30 pg/mL that is adequate for measuring tacrolimus concentration in oral fluid samples from transplant recipients. Full separation between tacrolimus and plasma phospholipids components was achieved in very short run time of 2.2 min. Very simple sample predations procedure was followed by extraction 50 µL of oral fluids with 100µL of acetonitrile.

Manuscript III in this manuscript, the method presented in manuscript II to quantify tacrolimus in oral fluids. It focused on investigating factors that may affect tacrolimus measurement in oral fluid, namely, sampling condition (resting, after mouth rinsing, and after give a saliva stimulant), sampling time, and blood contamination expressed as salivary transferrin level. The correlation between tacrolimus concentration in blood and oral fluids was investigated under these conditions. Correlation analysis revealed that samples collected after mouth rinse and at fasting provided better correlation in tacrolimus concentrations in blood and oral fluid.

Manuscript IV: Liquid chromatography tandem mass spectrometry methods was developed and validated according to current FDA Guidelines to quantify mycophenolic acid and its glucuronide metabolites in oral fluids, total concentration in plasma, and unbound fraction in plasma. Full separation of mycophenolic acid, metabolites, and plasma phospholipids was achieved within the total run time of 2.8 min.

Manuscript V: The assay described in manuscript IV was used to quantify mycophenolic acid and glucuronide metabolites in oral fluids. The aim was to

investigate factors that may affect mycophenolic acid and glucuronide metabolites concentration in oral fluid, namely, sampling condition (resting, after mouth rinsing, and after saliva stimulation), sampling time, and blood contamination expressed as salivary transferrin level. The result of this study indicated that the blood contamination had an insignificant effect on the concentration of mycophenolic acid and metabolites in oral fluids. In addition, a good correlation was observed between AUC_{0-12} of MPA in OF samples and unbound and total MPA. In contrast, a weak association was observed between MPAG concentrations in oral fluids with total and unbound plasma concentration.

Manuscript VI: PF-5190457 is a ghrelin receptor inverse agonist that is currently undergoing clinical development for the treatment of alcoholism. In this manuscript, the development and validation of a simple and sensitive assay for quantitative analysis of PF-5190457 in human or rat plasma and rat brain was described using liquid chromatography-tandem mass spectrometry. Full separation was achieved between the analyte and phospholipids of the three matrices within the total chromatographic run time of 2.2 minutes. The manuscript also identified and described the abundance of phospholipids contents of the three matrices. The developed method successfully used to quantify the analytes in the three matrices as part of pre-clinical and ongoing clinical studies.

ACKNOWLEDGMENTS

This dissertation would not be possible without the blessings of the Almighty God and guidance and support of several individuals. In particular, I would like to express my deepest appreciation to my major advisor Dr. Fatemeh Akhlaghi. Without her constant guidance, persistent help and encouragement this dissertation would not have been possible. Sincere thanks to her for giving me the chance to work under her supervision and for providing with knowledge that would help me to meet my long-term goals.

I would like to thank my doctoral committee chair Dr. Ingrid Lofgren and committee members, Dr. Sara Rosenbaum, Dr. Liliana Gonzalez, Dr. Ruitang Deng.

I am very much thankful to Dr. Reginald Gohh and Maria Medeiros RN, our research collaborators at Rhode Island Hospital for their persistent great effort in recruiting our study participants and other logistic work that helped us executing the clinical studies. Moreover, I would like to thank Dr. Deyu Li for his help in preparing manuscript VI.

I am very much indebted to the Libyan Ministry of Higher Education and Scientific Research for financial support throughout my study.

I am appreciative to all previous lab members for their help and support including Dr. Miroslav Dostalek, Dr. Ken Ogasawara, Dr. Ileana Ionita, Dr. Shripad Chitnis, Dr. Joyce Macwan, Dr. Amir Mohammadpour and Karen Thudium. I am also thankful to present lab members Sravani, Enoch Abdalla, Armin, Rohitash and Anita for the

enjoyable moments we have spent together. I deeply grateful to College of Pharmacy and the University of Rhode Island for providing me all necessary resources and the help of their personnel, especially Mrs. Gerralyn Perry and Kathy Hayes.

I am thrilled to express thankfulness to my dear wife Khouloud, for her love, patience, support and constant motivation. Not to forget my amazing kids Aser, Ahmed, Ayaat and Alaa for lovely and pleasurable moments that helped me to get through tough times.

DEDICATION

To My Deceased Mother and Father, May Allah Have Mercy upon Their Souls.

To My Wife and My Children

PREFACE

This dissertation was prepared according to the University of Rhode Island 'Guidelines for the Format of Theses and Dissertations' standards for Manuscript format. This dissertation consists of six manuscripts that have been combined to satisfy the requirements of the department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island.

MANUSCRIPT I: Alternative Matrices for Therapeutic Drug of Immunosuppressive Agents using LC-MS/MS.

This manuscript has been accepted for publication and submitted to *"Bioanalysis"* as a review article.

MANUSCRIPT II: Development and Validation of Sensitive and Selective LC-MS/MS Method for Quantification of Tacrolimus in Oral Fluid Samples from Kidney Transplant Recipients.

This manuscript has been prepared for publication and will be submitted to *"Journal of Chromatography B"*

MANUSCRIPT III: Therapeutic Drug Monitoring of Tacrolimus in Oral Fluids.

This manuscript has been prepared for publication and will be submitted to "*Clinical Pharmacokinetics"*

MANUSCRIPT IV: Development and Validation of Sensitive and Selective LC-MS/MS Method for Quantifying Mycophenolic Acid and Glucuronide metabolites in Oral Fluid, Plasma, and Plasma Ultrafiltrate.

This manuscript has been prepared for publication and will be submitted to *"Journal of Chromatography B"*

MANUSCRIPT V: Therapeutic Drug Monitoring of Mycophenolic Acid in Oral Fluid in Samples from Kidney Transplant Recipients.

This manuscript has been prepared for publication and will be submitted to "*Clinical Pharmacokinetics"*

MANUSCRIPT VI: Development and Validation of an UPLC-MS/MS Assay for Quantitative Analysis of the Ghrelin Receptor Inverse Agonist PF-5190457 in Human or Rat Plasma and Rat Brain.

This manuscript has been prepared for publication and submitted to "*Analytical and Bioanalytical Chemistry"*

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1 : MANUSCRIPT I

Submitted as review article to Bioanalysis

Alternative Matrices for Therapeutic Drug Monitoring of Immunosuppressive Agents using LC-MS/MS

Mwlod Ghareeb[1], Fatemeh Akhlaghi[1]
[1]Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, RI

Address correspondence to: Fatemeh Akhlaghi, PharmD, PhD, Clinical Pharmacokinetics Research Laboratory, Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, 495A College of Pharmacy, 7 Greenhouse Road, Kingston, RI 02881, USA. Phone: (401) 874 9205. Fax: (401) 874 5787. Email: fatemeh@uri.edu

Keywords
Transplantation, immunosuppressant, dried blood spot, saliva, oral fluid, lymphocytes, intra-tissue, therapeutic drug monitoring, LC-MS/MS.

Abbreviations
ACN: Acetonitrile, ASC: Ascomycin, C_0: pre dose concentration, C_2: Two post dose concentration, DBS: Dried blood spot, ESI: Electrospray ionization, EVE: Everolimus, HT: Hematocrit, LC: MS/MS: Liquid chromatography tandem mass spectrometry, LFB: Liquid finger prick blood sampling, LLOQ: Lower limit of quantification, ME: Matrix effect, MeOH: Methanol, MPA: Mycophenolic acid, MPAG: Mycophenolic acid glucuronide, OF: Oral fluid, PBMC: Peripheral blood mononuclear cells, P: gp: P: glycoprotein, PLN: Prednisolone, SIR: Sirolimus, SPE: Solid phase extraction, TAC: Tacrolimus, TDM: Therapeutic drug monitoring

Abstract

Immunosuppressive drugs used in solid organ transplants typically have narrow therapeutic windows and high intra- and inter-subject variability. To ensure satisfactory exposure, therapeutic drug monitoring (TDM) plays a pivotal role in any successful post-transplant maintenance therapy. Currently, recommendations for optimum immunosuppressant concentrations are based on blood/plasma measurements. However, they introduce many disadvantages, including poor prediction of allograft survival and toxicity, a weak correlation with drug concentrations at the site of action, and the invasive nature of the sample collection. Thus, alternative matrices have been investigated. This paper reviews tandem-mass spectrometry (LC-MS/MS) methods used for the quantification of immunosuppressant drugs utilizing non-conventional matrices, namely oral fluids, fingerprick blood, and intra-cellular and intra-tissue sampling. The advantages, disadvantages, and clinical application of such alternative mediums are discussed. Additionally, sample extraction techniques and basic chromatography information regarding these methods are presented in tabulated form.

Introduction

Therapeutic drug monitoring (TDM) is an integral part of immunosuppressive therapy following organ transplantation because of the narrow therapeutic index and high inter- and intra-subject variability of these agents [1-4]. The immunosuppressive agents used in solid organ transplant include cyclosporine (CsA), everolimus (EVE), mycophenolic acid (MPA), prednisolone (PLN), sirolimus (SIR) and tacrolimus (TAC) [5]. The incidence and severity of side effects of immunosuppressant agents correlate with a high exposure [5], while under-dosed patients can be at a greater risk for allograft rejection [1, 5]. Currently, whole blood or plasma samples obtained through venipuncture are used for routine immunosuppressive monitoring [5]. The limitations of venipuncture blood samples include the invasive nature associated with the sample collection and the weak correlation with the drug concentration at the site of action. In this review, these limitations and proposed alternative methods will be discussed.

Use of tandem mass spectrometry (LC-MS/MS) in drug monitoring

Advances in LC-MS/MS have enabled researchers to measure drug concentrations in limited sample volumes with adequate sensitivity, selectivity and robustness. This review will focus mainly on the use of LC-MS/MS in immunosuppressive agents in TDM using alternative matrixes, namely oral fluids (OF), dried blood spots (DBS), peripheral blood mononuclear cells (PBMC), and a biopsy sample from the implanted organ. Other techniques, such as high-performance liquid chromatography (HPLC)

and immunoassays, will be briefly discussed wherever significant findings have been reported.

The use of LC-MS/MS has long been a gold standard in pharmacokinetic studies [6], and it is becoming an increasingly used technique in clinical laboratories [7]. A reduced chromatographic run time and increased sensitivity are typically achieved using ultra-performance liquid chromatography (UPLC) and newer stationary phases [8,9]. LC-MS/MS has enabled researchers to quantify lower drug concentrations in small blood sample volumes (i.e., 4-10 μL) [10-15] with higher specificity in comparison with immunoassays [16-20]. In addition, LC-MS/MS allows the simultaneous quantification of more than one analyte and/or metabolite [9, 21] with different physiochemical properties with a high degree of sensitivity and selectivity [22].

LC-MS/MS is a system that combines high-performance chromatography (HPLC) with mass spectrometry (MS). Three atmospheric pressure ionization (API) techniques, namely electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI), and atmospheric-pressure photo-ionization (APPI), are typically employed [23]. These techniques provide highly precise quantitative analysis with minimal sample preparation of complex samples such as blood, plasma and OF [22, 24, 25]. ESI technique, most commonly used in quantifying polar to ionic compounds, and in metabolomics and proteomics studies [23]. The main challenge that may hinder the LC-MS/MS method development is the **matrix effect** (ME), which may produce erroneous results [26, 27]. Proper cleanup of samples [26], the use of a **deuterated**

internal standard [21], and chromatographic separation of analytes from regions of ion enhancement or suppression can mitigate/eliminate the effect of ME [28].

1. Oral fluids as a matrix for therapeutic drug monitoring

Oral fluids have been a subject of interest as an alternative medium to venipuncture blood [24, 25, 29-39]. The main advantage of OF sampling is the noninvasive sample collection, permitting more frequent sampling [40] and allowing more convenient self-sampling [41]. Moreover, OF sampling offers a significantly lower cost per sample [41, 42]. In addition, the drug portion measured in the OF represents the free drug concentration [41, 42] (Figure 2). Given that the free drug concentration is responsible for the pharmacological and toxicological effects [4, 43, 44], measurement of the drug concentrations in OF may provide a better prediction of clinical outcomes and toxicity [34,45]. Therefore, salivary drug level measurements are much easier and faster than quantifying the free drug concentration in plasma [25, 38].

Drugs enter the OF mainly via passive diffusion [35]. Thus, physiochemical properties, including protein binding, ionization, lipophilicity, and molecular weight, are important determinants for the entry of a drug into the OF [35, 45]. The ability of a drug to diffuse and equilibrate between the plasma and tissues is governed by its free fraction [[35,45,46]. According to Lipinski's rule of five, a molecular weight < 500 is a prerequisite for good absorption/permeability [47]. However, despite its large molecular weight (1202.6 g/mol), the total cyclosporine (CsA) concentration in both blood and OF has shown a reasonable correlation (r=0.695) [39]. Blood capillaries contain pores that are sufficiently large to allow molecules with a molecular weight <1000 to permeate [45]. Because of their large size, drug-protein complexes are

prevented from crossing capillaries, and only the unbound drug enters the OF [34]. The salivary flow rate (see section 1.1.1), pH and pathophysiological conditions of the oral cavity are also important physiological factors that affect the movement of a drug between the plasma and the OF [48]. The pH of a medium influences the drug distribution by altering the unionized portion of a drug [29,34,35,45,46]. The degree of ionization of a drug is determined by its pKa (the pH at which 50% of the drug is found in ionized form) and the pH of the medium [33]. Theoretically, basic drugs with pKa values less than 5.5 and acidic drugs with pKa values greater than 8.5 are not affected by changes in salivary pH (5.8-7.8) [45,48]. Under these conditions, drugs predominantly exist in unionized form, therefore; they have higher lipophilicity and consequently cross biological membranes more easily [29,35]. The chemical structure and physicochemical properties of immunosuppressive agents are presented in **Figure 1** and **Table 1,** respectively.

Recently, a Saliva Excretion Classification System has been proposed to predict the ability of drugs to diffuse into the OF [49,50]. This system is based on the estimated effective intestinal permeability and the percentage of the free fraction. According to the authors, high drug permeability and/or high percentage of free fraction are required to ensure the smooth movement of a drug between the plasma and OF. Based on the logD value at pH 7.4, all immunosuppressive agents have high lipophilicity (Table 1), and therefore high permeability is predicted despite the low free fraction. A low free fraction thus will be the rate-limiting factor for penetration of drug into saliva making saliva a suitable specimen to measure the unbound concentration of immunosuppressive agents.

1.1. Oral fluid collection techniques and storage

1.1.1. Resting vs. stimulated OF sampling

The concentrations of certain drugs in the OF are affected by the salivary flow rate [29,35,48]. Stimulated OF has less contact time in comparison to resting OF, consequently reducing the influence of tubular re-absorption and secretion [29,48]. Stimulation may alter the salivary composition and pH [51], thereby may affect the partitioning of drugs between the OF and plasma [52] by modifying the ionized portion. Changing the salivary flow rate alters the correlation between the plasma and OF drug concentrations of some drugs but has little to no effect on others [29,48]. Acidic drugs mainly exist in non-ionized forms at a lower salivary pH, which allows better correlation with the plasma concentration [33]. In contrast, basic drugs tend to accumulate in acidic saliva because they exist predominately in the ionized form, which limits their movement across biological membranes [29] (Table 1). Using Henderson-Hasselbach equation, it can be predicted that except for MPA, all immunosuppressive agents are mainly (> 99%) unionized at pH 7.4; therefore, their high lipophilicity should lead to a good agreement between blood and OF concentrations. Conversely, >99% of MPA exist as ionized that should theoretically limit the ability of MPA to move through biological barriers. However, published reports [25,38] indicate that MPA concentration in OFs associates well with the plasma concentration of MPA.

In addition, food stimulates protein-rich OF, compared with other stimuli that produce protein-poor OF [33]. No published studies have investigated the effects of salivary stimulation on the distribution of immunosuppressive agents into the OF.

1.1.2. Influence of oral fluid collection device materials

Depending on the analyte of interest, appropriate collection devices should be chosen, and OF collection protocols should be optimized [53]. In a study reported by Groschl et al. [53], the suitability of different devices for OF sampling of several endogenous substances and chemical entities were evaluated. Devices for collecting peptides, proteins and steroids that are made of polyester, polyethylene and cellulose were found to be superior to those made of cotton. Devices consisting of polyester and polyethylene showed excellent stability for small molecules (e.g., antidepressants, theophylline and caffeine). With a few exceptions (phenobarbital, ethosuximide and amylase), cotton pads exhibited very poor recovery. Salivette® (Sarstedt) devices consisting of cotton, polyester or polyethylene roll were highly rated by patients and investigators based on their ease of use and practicality. The OF collection methods used in the immunosuppressive agent quantification assays are shown in **Table 2**.

The adsorption of TAC into plastic materials, including polyolefin and polyvinyl chloride used in making central venous catheters, has been reported [54]. However, a recent study showed that the stability of TAC was not compromised when it was stored in either glass or plastic containers [24]. The yield of TAC obtained from OF samples with passive drool and polypropylene Salivette® devices was also studied. A modest correlation ($r = 0.57$) was reported in TAC concentrations in drool and

Salivette® samples [24]. Although minimal to no interaction was observed between CsA and plastic/glass materials used in the manufacture of blood collection tubes, the adsorption of CsA into peripheral and indwelling catheter sites has been reported [55]. To prevent non-specific binding and to minimize the risk of adsorption, siliconization (i.e., the application of a thin layer of highly hydrophilic material) of the OF collection and storage containers may prove to be beneficial [39]. To date, no studies have investigated the suitability of different OF collection devices or the optimal collection conditions for the immunosuppressive agents used in solid organ transplants. For more information on OF collecting devices, the reader is referred to other published papers [53,56,57].

1.2. Sample preparation and extraction

The mucopolysaccharide content of OF may interfere with the accuracy of pipetting [58]. Sample homogenization aids in breaking down salivary proteins and improving extraction yields [38]. Subjecting OF samples to freeze and thaw cycles followed by centrifugation facilitates sample processing and breaks down mucopolysaccharides [58]. Simple pre-analysis treatment and protein precipitation using 2-3 volumes of acetonitrile (ACN) has been shown to provide sufficient sample cleanup and good recovery [24-26]. Some methods employ more labor-intensive techniques, including SPE and drying for sample cleanup [37-39].

1.3. Blood contamination of oral fluid

Predicting the effect of mouth injuries based on the concentration of endogenous compounds in OF is not straightforward. For example, the presence of a low

concentration of blood in the OF does not alter the cortisol concentration if no visual discoloration is detected [58]. In contrast, the validity of salivary testosterone measurements can be compromised by even minimal blood contamination from micro-injuries caused by routine teeth brushing, as detected by the transferrin immunoassay (Salimetrics LLC, State College, PA) [58]. Therefore, the effect of OF blood contamination on the accuracy of each analyte should be investigated.

To analyze the possible effect of blood contamination on MPA and TAC, the salivary levels were investigated. Mendonza et al. [38] utilized a Salimetrics **transferrin** kit to detect the presence of transferrin and excluded samples with a transferrin level >1 mg/dL. Fasting OF samples displayed significantly higher transferrin levels than non-fasting OF samples, and this difference was accompanied by an elevated MPA concentration. In another study [24], the influence of salivary blood contamination on the TAC level was investigated. When 1 mL of blank OF samples spiked with different volume of blood (<1, 2, 5, and 10 μL) contained TAC (11.2 μg/L) were analyzed, only samples that were spiked with 2, 5, and 10 μL of TAC displayed visual signs of blood contamination together with proportional increases in TAC concentrations up to 28%. Thus, visual inspection might be sufficient for sample exclusion due to blood contamination for TAC.

1.4. Measurement of immunosuppressive agents in oral fluids

In the following paragraphs, the physiochemical characteristics of immunosuppressive drugs will be presented, and LC-MS/MS methods that utilize OF will be discussed.

1.4.1. Cyclosporine

Cyclosporine is an extremely lipophilic compound that is mostly distributed in plasma lipoproteins and blood cells [44]. Measurement of unbound fraction of CsA by equilibrium dialysis is difficult and time consuming. Because of extreme lipophilicity, CsA binds non-specifically to Teflon dialysis cells resulting in low yield and prolonged dialysis time. As a result, unbound fraction measurement requires the use of custom-made stainless-steel equilibrium dialysis devices [44]. Moreover, all methods reported to date have utilized radiolabelled cyclosporine as tracer possibly because of lack of sensitivity of analytical methods.

The degree of binding to plasma proteins is influenced by the time after transplantation [60], drugs that modulate the lipid profile [44,59], nutritional status [60], and clinical conditions [60,61]. Cyclosporine partitioning between the blood and plasma depends on the drug concentration, hematocrit (HT), plasma lipoprotein level and temperature. Therefore, whole blood is the recommended matrix for CsA therapeutic drug monitoring [60]. The outcome of immunosuppressant therapy with CsA is improved by a higher free fraction percentage [62]. There is a high variability in the free fraction of CsA with a mean ± SD of 1.53 ± 0.38% in the lung and heart transplant recipients [44] and a range from 0.5 to 4.2% [61]. The ease with which cyclosporine crosses biological membranes and enters the OF is attributed to its lipophilicity. CsA was the first immunosuppressant agent studied in OF by radioimmunoassay [36]. A good correlation was reported ($r=0.68$) between the OF and the total cyclosporine serum level in samples from 38 renal transplant recipients. Mendonza et al. [39] published the first and, to date, the only method used to measure

CsA concentrations in the OF by LC-MS/MS following SPE for sample cleaning (**Table 2**).

1.4.2. Tacrolimus

Tacrolimus is a highly lipophilic compound (**Table 2**) with a plasma free fraction of approximately 1% [3]. The unbound fraction is significantly affected by changes in plasma lipoprotein concentrations after liver transplantation [43], which may lead to incidences of rejection and/or toxicity [43,63]. There is only one published method for the utilization of the OF matrix for TAC quantification [24] (**Table 2**).

1.4.3. Mycophenolic acid

The unbound fraction of MPA ranges from 1 to 2.5% [4]. In patients with severe renal impairment, the concentration of the major MPA metabolite, MPA-glucuronide (MPAG), may increase up to 3-6-fold. This increase in MPAG leads to displacement of MPA from its binding sites [4], and as a result, the MPA-free fraction may increase up to 7% [4]. Mycophenolic acid has a low molecular weight and lipophilic nature (logD 0.76 at pH 7.4) (**Table 1**). These characteristics make MPA a suitable candidate for TDM in OF. LC-MS/MS is used to quantify MPA in negative [38] and positive [25,37] ESI modes. In a recent paper [37], MPA and MPAG were quantified simultaneously with 82.1% and 65.7% recovery, respectively. It must be noted that MPAG is subject to in-source conversion to MPA. This phenomenon is observed as small peaks in the MPA chromatogram channel with the same retention time as MPAG [25,38,64]. Therefore, the chromatographic separation of MPA and MPAG peaks is necessary to avoid overestimating the parent drug concentration.

1.4.4. Prednisolone

Prednisolone (PLN) is a synthetic glucocorticoid with an unspecific mechanism of action [65]. Prednisolone is widely prescribed as a part of immunosuppressive therapy regimens in solid organ transplantation [66]. The free fraction of PLN increases in certain clinical conditions such as diabetes [67]. In addition, the free fraction is dose-dependent and exhibits circadian variability (approximately 22% higher in the morning) [68]. The PLN plasma unbound fraction demonstrates a high correlation with the salivary level and a lower correlation with the concentration of the pro-drug prednisone (PN) [69,70]. Total and free concentrations of PLN+PN in the OF and plasma display an excellent association [70] (**Table 2**).

Some studies [24,39] have focused on finding an association between total drug concentrations in the blood and OF. Because the drug fraction in OF theoretically represents the unbound portion, a good association with the free fraction in the blood should be pursued. The total drug concentration may not correlate very well with the free fraction [4,43]. However, OF sampling may be considered a non-invasive alternative to venous blood sampling if a good correlation between total blood and OF drug concentrations is established.

2. Dried blood spot and liquid fingerprick blood sampling

Dried blood spot (DBS) and **liquid fingerprick blood (LFB)** sampling are other techniques that have benefited from the introduction of LC-MS/MS [12-14,71-77]. The first report of the use of fingertip blood to measure an immunosuppressive agent was published in the late 1980s [78]. The radioimmunoassay (RIA) technique was

utilized to quantify CsA in 20-µL blood samples obtained from the fingertips of renal transplant recipients with a lower limit of quantification (LLOQ) of 62.5 µg/L. Fingerprick sampling is much less invasive than venipuncture and offers the possibility of home self-sampling at the patient's convenience [79]. However, adequate patient training might be needed for optimum sample collection [80]. Additionally, proper sample handling and storing after collection are required to avoid deterioration and to ensure stability during mailing and transportation [73,79].

2.1. Sample collection

After cleaning the fingertip with a suitable disinfectant [11,14,79,81], a small laceration is made using spring-loaded lancets that are designed to minimize pain and discomfort [73,77,79,82]. A fingerprick blood sample is processed either as a dried blood spot (DBS) (**Table 3**) or in liquid form (LFB) (**Table 4**).

In the DBS technique, blood samples are either applied directly from the fingertip after discarding the first drop [71,73,74,76,79,82]; or the blood is collected using a collecting device from the fingertip or venipuncture is pipetted onto a predetermined circular area of a special filter paper [15,77,83]. The latter approach guarantees the application of a precise amount of blood sample to the filter paper. However, this additional step may make home self-sampling less appealing [15]. In addition, capillary self-sampling may result in a significantly different result from sample collection by healthcare professionals [15]. Liquid fingerprick blood sampling involves the direct extraction of blood samples in liquid form, which are collected

using EDTA-containing devices such as Microvette™ [12,15] or Microtainer™ tubes [13].

2.2. Extraction procedure and recovery of DBS and LFB sampling

A disc of the blood spot with a diameter between 4 and 8 mm is removed using a special puncher. The sample extraction ranges from simple vortex mixing [15,75,82] to ultra-sonication at temperatures of up to 80 °C [71,72,77,83]. The different pre-treatment conditions used for the samples result in significant differences in the final yield (**Table 3**). The applied blood volume, card type, punched area, and hematocrit (HT) may also play important roles in the extraction recovery and method reproducibility [84,85]. Therefore, these variables should be examined for the analyte of interest, and corrections should be applied if necessary and feasible [86]. LFB samples are pre-treated with a zinc sulfate solution (0.1- 0.4 mol/L) followed by protein precipitation with ACN and centrifugation [11-14].

2.2.1 Effect of blood volume

A good precision of the estimation of CsA, SIR, and TAC (CV=4.3-13.5%) was produced using a 25-100-µl blood drop on a Whatman 903 card with an 8-mm punch size [75]. A different study [87] reported that 20 µL of blood was enough to fill the designated area on the Whatman 903 card. In contrast, another study [71] used the same type of filter paper and punch size reported that drops with a volume of 20 µL were insufficient to fill the pre-determined area. The discrepancy between the two studies could be attributed to differences in the HT values of the blood samples used.

2.2.2. Influence of the type of sampling card

A Whatman Protein saver 903 card (LifeSciences GH) [88] is the most commonly used card for immunosuppressive drug testing (**Table 3**). This card is additive-free and made from 100% pure cotton linters [89]. Whatman FTA and FTA Elute are high quality papers that are chemically treated to provide cell lysis, protein denaturation and prevention of microorganism growth [89]. Whatman 31 ET CHR (chromatography/ethyl acetate) cards are intended for electrophoresis applications of large molecules and are also used in immunosuppressant drug DBS testing [88]. Finally, Ahlstrom 226 (PerkinElmer) is another additive-free sampling card that consists of 100% pure cotton linter and is validated for even and uniform sample distribution [90].

There are no significant differences between Whatman 31 ET CHR and Whatman FTA cards at the method validation level for CsA, EVE, SIR and TAC [86]. Heinig et al. [87] compared MPA and MPAG metabolite recovery using five different cards. There were lower recoveries of MPA and metabolites from Whatman FTA-DMPK-C and FTA-DMPK-A than from Ahlstrom 226, FTA-DMP-B, and Whatman FTA elute cards. In addition, poor reproducibility (CV=17-26%) was observed for FTA-DMPK-C and FTA-DMPK-A. Although 20 µL of blood was sufficient to fill the designated area on the Whatman 903 card, there was a visible clear area on the Ahlstrom 226 card.

2.2.3. Effect of the punching location

The distribution of analytes may differ between the center and the outer area of the spot due to the chromatographic properties of the DBS sampling card [85]. The disc obtained from punching close to the spot edges on Ahlstrom 226 cards produces 30% higher MPA and metabolite (MPAG) concentrations than the concentrations determined from central punching [87]. In contrast, the concentrations of MPA and its metabolites at the edges were lower on FTA Elute and DMPK-B (4–10% and 14–19%, respectively) [87]. Consistency of the punching location helps to improve the reproducibility [87] and application of a larger spot than the size of the punched disc ensures sampling from the center of the spot [71].

2.2.4. Effect of hematocrit

Normal HT values range from 42 to 52% in males and from 37 to 48% in females [91]. Samples from patients with a high HT create drops that are more viscous and have smaller volumes [92]. Furthermore, a drop with a high HT produces less dispersion on the filter paper, and a larger volume is required to fill the same area [86]. Consequently, the concentration of certain analytes can be overestimated [77,85,86,92]. A high HT has been reported to increase the MPA content by approximately 10% [92]. Similar findings have been reported for CsA from blood samples with high HT values (0.72%), demonstrating an approximately 10-14% higher CsA concentration [77]. Conversely, in blood samples with HT levels less than 0.20, the CsA concentration was reduced by approximately 9-12%. The normalization of individual HT values with an average HT value of venous blood obtained from the

precipitating individuals is recommended to minimize the effect of variability in HT on the finalized results [71,77,91,92]. Using this approach, the calculated recovery in samples with low HT improved to 112.4 and 97.0 for low (39.4 μg/L) and high (590 μg/L) CsA concentrations, respectively, compared to less than 85% for non-normalized HT values [71]. The effect of HT on the recovery of EVE, SIR and TAC appears to be minimal [71].

Recently, a new technique has been proposed to overcome variability in volumes of blood samples applied to the filter paper arises from differences in HT value [93,94]. This utilizes simple and practical procedures using volumetric absorptive microsampler devise (VAMS). It consists of porous absorbent polymeric tip capable of absorbing more precisely 10 μL of blood utilizing capillary force.

2.3 Matrix effect

The extracted matrix from DBS appears to have a negligible effect on ME [71,76,77,82,87]. The degree of interference of blood components may depend on the type of sampling card used. For example, interfering residue is less pronounced in ethanolic extract from samples prepared on the Whatman FTA elute and FTA DMPK-A card than from samples prepared on the Ahlstrom 226 card [87]. However, remains were further reduced after proper sample cleaning using SPE [87]. Using MeOH: water (80:20, v:v) as an extracting solvent from the Whatman 31 ET CHR and Whatman FTA cards, only CsA showed a significant ME; no interference was observed with EVE, SIR or TAC [86]. The ME effect on CsA was diminished when a deuterated internal standard was used.

2.4. Stability

Despite the use of the same DBS collection paper (Whatman 903), a discrepancy in stability has been reported, especially for CsA (**Table 3**). Leichtle et al. [15] have examined CsA stability in DBS. After the application of capillary venous blood (about 4 μL), the card was allowed to dry for two hours, and the CsA was extracted from a 4-mm disc. Samples collected with capillary devices with or without EDTA were stable for up 12 hours at 8 and 20 °C; the concentration decreased significantly by 24 hours. In contrast, no identifiable changes in the blood samples processed in liquid form were observed. Shorter stability time in the DBS samples compared to the capillary blood samples, may indicate an insufficient drying time (2 hours) and/or poor storage conditions and handling [89].

In another study [77], CsA concentrations were measured in dry blood spots prepared by pipetting EDTA venous blood samples (50 μL) onto filter paper that was allowed to dry overnight at room temperature. The extracted CsA from an 8-mm disc was stable for 17 days at ambient temperature and for up to 45 days at 4 °C. Finally, a recent study [75] reported that CsA extracted from an 8-mm disc prepared using 50 μL EDTA venous blood dried for 3 hours at room temperature was stable for up to 5 days at 60 °C. The only noticeable differences seemed to be the drying time and sample volumes, which were approximately 12-fold higher in the latter two studies [75,77] (see **Table 3**).

Tacrolimus that was measured in EDTA venous blood (50 μL) applied immediately onto a filter paper and dried at room temperature for 3 hours showed stability for up to

5 days at 60 °C, and SIR was stable for the same period of time at 37 °C [75]. Tacrolimus in fingertip blood samples applied directly onto the filter paper also showed stability for up to 7 days at 37 °C [82]. In addition, EVE appeared to be stable for up to 3 days at 60 °C and for 32 days at 4 °C [76]. Fingertip DBS samples of CsA, EVE, SIR and TAC have been reported to be stable for up to 5 months at 2 to 8 °C when the blood was applied directly onto the filter paper [71].

Cyclosporine A in LFB blood samples collected in Microvette devices containing EDTA were found to be stable for 5 days after mail delivery [12]. Tacrolimus [14, 79], EVE [76], and CsA [12] DBS samples seemed to be stable during mailing and transportation, supporting LFB and DBS home sampling.

2.5. Patient preference

Self-fingerprick sampling is well tolerated with no serious discomfort as reported by children [14,81] or adult transplant patients [12,79,95]. In solid organ transplant patients, LFB was preferred (60%) over venipuncture sampling, and approximately 68% of patients favored the use of DBS over LFP sampling (18%) [15]. The sampling process for LFB may be troublesome for some patients and therefore may produce poor sampling [12,15]. Nonetheless, unsupervised capillary and DBS self-sampling can be improved by providing brief instructions or over-the-phone consultation [12,73].

2.6. Clinical application of DBS and LFB

The mean difference in CsA concentrations is significantly higher in DBS prepared from capillary tube-collected fingertip blood than from venous blood at C_0 and C_2 [15]. Despite the low recovery of EVE from DBS (76.5%), the concentration of EVE in DBS was slightly higher than in venous blood. The concentrations of EVE in DBS samples prepared by patients and in the laboratory were very similar [76]. Cheung et al. [79] used DBS to estimate TAC exposure ($AUC_{0–12}$) utilizing a limited sampling strategy (C2 and C4) in 36 kidney transplant recipients. The dried blood spot results showed a high correlation with the results obtained from analyzing venous blood samples (r^2= 96, P<0.001). The calculated $AUC_{0–12}$ mean difference between DBS and venous samples was less than 7.6%.

A high correlation between venous and fingertip samples is expected because both represent whole blood. However, a statistically significant higher TAC has been reported in LFB samples compared to venous blood, but the mean difference was clinically insignificant (0.29 ng/mL, 95% CI 0.09–0.49), and a good correlation was reported (r^2 =0.845) [14]. In contrast, the CsA venous blood level was statistically significantly higher than in LFB [11]. The mean difference was 9.5 ng/mL (95% CI 0.8-18.2 μg/L, P<0.03), however, a strong association was also reported between venous and LFB samples (r^2 = 0.96, P < 0.001).

Because fingertip sampling utilizes whole blood, a lack of correlation is expected between the obtained levels of immunosuppressive agents in DBS or LFB and their levels at the site of action (see sections 3 and 4). However, the relative ease of DBS

and LFB sampling compared to venipuncture, the possibility of home self-sampling, and the stability during storage and transportation suggest that both of these techniques have the potential to replace venipuncture in TDM.

3. Intracellular concentration

Despite maintaining a satisfactory blood level of immunosuppressants through intensive TDM, rejection rates still remain between 8-15% [96], which necessitates the need to develop a new approach that could further reduce the rejection rate.

To prevent allograft rejection resulting from suppressing the immune system, immunosuppressants must first enter lymphocytes [97-99]. In heart transplant recipients, there is a greater incidence of rejection associated with a higher peripheral blood monocyte cell (PBMC) count [100]. Lymphocytes express P-glycoprotein efflux transporter (P-gp), which is also known as multidrug resistance protein 1 (MDR1) encoded by the *ABCB1* gene [101-103]. This transporter is responsible for moving xenobiotics from the intracellular to the extracellular environment [103]. As a result, the intracellular level of P-gp substrates can be affected by genetic polymorphisms in the coding gene of P-gp, altering the immune system response [103,104]. Both CsA and TAC are well-documented substrates of P-gp [104-106]. In vitro data indicate that SIR is a substrate and a weak inhibitor of the P-gp transporter [107-109], while EVE has shown a weak inhibitory effect on P-gp [109]. Higher incidence of rejection is proportionally correlated with higher expression of MDR1 gene on PBMCs obtained from heart [100] and liver [110,111] transplant recipients who have been prescribed CsA or TAC. The levels of immunosuppressants in lymphocytes, including

CsA [104,112-116], TAC [104-106,113,114,116-124], SIR [125] and EVE [126], have been investigated in solid organ transplant patients (**Table 5**).

There is a histologically and clinically proven rejection associated with a lower level of TAC in PBMCs measured at day 7 post-transplantation in liver transplant recipients [117]. No correlation between whole blood and PBMCs' tacrolimus concentrations in heart (r^2 = 0.259; P=0.183) and liver (r^2 = 0.0142; P=0.42) transplant recipients has been reported [106,113]. Contradictory findings have been reported for CsA. A study by Gustafsson et al. [119] involving heart transplant recipients co-treated with MPA reported a high correlation (r^2=0.98, P<0.001) between CsA concentrations in two hours post-dose (C_2) whole blood samples and lymphocyte $AUC_{0–12h}$ exposure (expressed as ng*h/10^{-6} cells). In contrast, a poor correlation was reported in patients co-treated with EVE (r^2 = 0.24, P = 0.18). The authors suggested that the difference between the two groups could be attributed to the inhibitory effect of EVE on P-gp, leading to modulation of intracellular CsA levels. A poor correlation (r^2 = 0.055, P = 0.35) in CsA levels in matched pre-dose (C_0) samples of blood and intra-lymphocytes from heart transplant patients was also reported in a recent study by Robertsen et al. [112]. Robertsen et al. suggested that the high correlation detected in the study by Gustafsson et al. could be attributed to the use of C_2 blood concentrations, which are known to correlate better with blood $AUC_{0–12h}$ than C_0. In addition, another study reported a weak correlation between blood and PBMC $AUC_{0–12h}$ in healthy volunteers following a single dose of CsA (Spearman, r=0.09, P=0.71) [105]. Slightly better correlation was observed in C_0 samples from stable renal, liver, and lung transplant recipients (r = 0.30, P<0.001) [104]. A study by Falck et al. [120] involving kidney

transplant recipients reported that, patients who experienced rejection displayed significantly lower CsA intra-lymphocyte $AUC_{0–12h}$ exposure compared to the non-rejection group (P = 0.004), despite identical CsA blood levels. The level of CsA in lymphocytes started to decline 7 days prior to clinical signs of rejection. The difference in intracellular concentrations between the two groups reached statistical significance (P = 0.014) three days before showing clinical signs of rejection. Regarding EVE, a poor correlation between blood and PBMCs concentrations has been reported (r = 0.32) [126]. Finally, in heart transplant recipients, a higher incidence of rejection is associated with elevated PBMC counts in patients who are receiving a triple drug regimen, including azathioprine, cyclosporine and steroids [100].

3.1. Effect of genetic polymorphisms of *ABCB1* on intracellular immunosuppressants concentrations

A recent report involving 90 liver transplant patients reported the involvement of genetic polymorphisms in P-gp transporters in modulating the concentration of TAC in intra-lymphocytes at day 7 and steady-state [106]. Absolute, dose normalized, and PBMC/blood TAC concentrations were 1.4 times higher (P<0.002) in carriers of the mutant 1199G>A allele than in non-carriers. Additionally, carriers of the mutant alleles 3435C>T and 2677G>T/A showed a 1.3-fold higher intracellular TAC concentration (expressed in the geometric mean) compared to individuals with homozygote wild type alleles (P values = 0.0089 and 0.0122 for 3435T and 2677T/A, respectively). A similar effect of genetic polymorphisms in the P-gp transporter on CsA has been reported in 3435T carriers among 64 stable renal, liver and lung

transplant recipients [104]. Carriers of 3435T showed an increase in intracellular CsA concentrations of 1.7 times (P = 0.04) compared to wild type (P = 0.02). However, the opposite findings have been reported in 1199A carriers, in whom intracellular concentrations of CsA were 1.8 times lower (P = 0.04) compared to wild type. The 2677T polymorphism did not affect the intracellular concentration of CsA.

CYP3A-metabolizing enzymes are also expressed in lymphocytes [127,128]. CYP3A enzymes are polymorphic [129-132], but the intracellular TAC concentration is unlikely to be influenced by genetic polymorphisms in CYP3A enzymes [106].

In summary, an adequate intracellular concentration of immunosuppressant drugs is pivotal for proper allograft maintenance. Monitoring the intracellular levels of immunosuppressants and detecting any changes in exposure could serve as an early warning call prior to the clinical manifestation of toxicity or rejection.

3.2. Sample preparation and extraction of immunosuppressants from PBMCs

The volume of whole blood needed to prepare PMBCs ranged from as low as 1.5 mL to as high as 10 mL (**Table 5**). To prevent immunosuppressant efflux from PBMCs during sample preparation, it is crucial to add a P-gp inhibitor such as verapamil or to perform the preparation procedures at 4 °C. The main limitations of intracellular drug concentration quantification methods the invasive nature of obtaining blood samples and the labor-intensive sample preparation procedures, which involve cells counts, drying and reconstitution and solid-phase extraction.

4. Intra-tissue concentration

Early reports on the measurement of intra-tissue concentrations of immunosuppressive agents date to the early 1990s [133-135] (. In those studies, HPLC and enzyme immunoassay (EIA) methods were used to measure CsA and TAC tissue concentrations, respectively. Recently, there has been a renewed interest in utilizing biopsied tissue from transplanted heart, kidney and liver allografts [112,136-139] (**Table 6**).

Post-mortem examinations have revealed that CsA and its metabolites accumulate rapidly in tissues after administration [133]. Measured using HPLC, the total concentration of CsA and its metabolites reached levels that were 53-fold higher in organs and tissues than in whole blood [133]. Tissue CsA concentrations were highest in the pancreas, followed by the spleen, liver, kidney, lung, and heart. In a recent study [137], analyses of CsA concentrations in kidney biopsies utilizing LC-MS/MS confirmed previous study findings and demonstrated a CsA concentration that was approximately four times higher in kidney tissue than in whole blood. A poor correlation between CsA in blood and liver biopsies obtained from liver transplant recipients has been reported. Sandborn et al. [135] showed no differences in the blood concentrations of CsA in patients with and without rejection. In contrast, the hepatocytes level of CsA was approximately two times higher in patients without autopsy-proven rejection compared to the rejection group. Moreover, little to no correlation in CsA concentrations has been reported between the blood and the kidney ($r = 0.168$, $P>0.05$) [137] or endomyocardial biopsies ($r^2 = 0.029$, $P = 0.48$) [112].

Similar findings have been reported for TAC in liver transplant recipients [134]. There was a trend detected in TAC hepatocyte concentrations based on the condition of the allograft. The highest TAC levels were found in liver biopsies from patients with no detected rejection (median = 144 ng/g), followed by patients with no current signs of rejection but with subsequently demonstrated rejection (median = 87 ng/g). The lowest concentrations were detected in patients with current rejection (median = 48 ng/g). In contrast, all three groups showed no significant differences in plasma concentrations (median = 0.9, 0.9 and 0.6 μg/L, respectively). Similar results were found in recent studies using LC-MS/MS to evaluate the correlation between TAC concentrations in C_0 blood samples and liver tissues on day 5 and 7 after transplantation [117,136]. Concentrations of TAC in hepatocytes displayed a significant first-order exponential correlation r^2 = 0.720-0.96 with Banff scores (histological marker of rejection) [117,136]. Higher concentrations of TAC in liver tissues were associated with lower Banff scores and consequently fewer episodes of rejection [117,136]. In contrast, a poor correlation has been reported between Banff scores and the blood level of TAC (r^2 = 0.0281) [117]. In kidney transplant recipients (2 patients) [138], a decrease in TAC was observed in tissue and C_0 whole blood over time (16-300 days) but, there was no correlation between the two measurements.

Only one published study investigated the intra-tissue concentrations of MPA. This study was performed using biopsies obtained from four kidney transplant patients. The authors were unable to determine the association between plasma and intra-tissue concentrations of MPA [139].

4.1. Effect of *ABCB1* gene polymorphisms on tissues concentrations of immunosuppressive agents

The inter-subject variability of P-gp substrates in tissues may be the result of genetic polymorphisms in P-gp transporters. Indeed, significantly higher TAC concentrations have been found in hepatic tissue from patients carrying alleles with reduced activity [140]. There were significantly higher hepatic tissue TAC concentrations, expressed as the geometric mean of the dose-normalized hepatic concentration, in carriers of the reduced-activity 1199A allele (1199A) than in non-carriers (P=0.036). Correspondingly, hepatic tissue obtained from carriers of the 236C>T and 2677G>T/A alleles demonstrated a higher TAC concentration, expressed as the geometric mean of the hepatic concentration (P = 0.014 and 0.035, respectively). Finally, although CYP3A-metabolizing enzymes are expressed in hepatic tissues, they have no effect on hepatocyte TAC concentrations [140]. In summary, the blood concentration of immunosuppressive drugs in solid organ transplant recipients is a poor predictor of intra-hepatocyte levels.

5. Conclusions and future prospective

Optimal exposure to immunosuppressant agents is required to improve allograft survival and reduce toxicity. Despite its limitations, venous blood remains the recommended medium for TDM of immunosuppressive agents. Limitations include the lack of association with *in situ* concentrations and the invasiveness of the sample collection. The introduction of LC-MS/MS into clinical practice has further encouraged investigating alternative matrices to overcome these limitations.

Intracellular and intra-tissue immunosuppressant measurements are proven predictors of allograft survival and toxicity. Nonetheless, the complexity associated with obtaining and processing samples makes these approaches impractical for routine TDM. The area under the concentration-time curve (AUC) and maximum concentration (C_{max}) are the best parameters to estimate because they correlate better to the clinical outcome and toxicity when whole blood is used [1]. Unfortunately, the estimation of AUC and C_{max} requires multiple sampling over a dosing interval of up to 12 hours, which is unsuitable for routine TDM. The relatively simple sample preparation procedures involved with fingerprick sampling offer a less invasive alternative and the possibility of multiple self-home samplings. However, because finger sampling utilizes whole blood, it provides drug measurements that are poorly related to the concentration at the site of action. Finally, OF sampling provide a simple process to quantify the free drug concentration in non-invasively collected samples that can be easily collect by patients at home. Recently, multiple sampling of oral fluid has been successfully used to individualize glucocorticoid replacement therapy in patients with Addison's disease [141]. If a good association is established between the drug concentration in OF and the sites of action or blood-free fraction, OF has the potential to replace blood drug measurements, making repeated sampling and calculations of AUC and C_{max} for the TDM of immunosuppressant agents feasible.

Table 1-1. Physiochemical properties of immunosuppressant drugs measured in oral fluids

Drug	Free fraction (%)	*Molecular weight (g/mol)	Chemical formula	¥*LogP	*LogD (pH 5.5)	*LogD (pH 7.4)	§pka	*Polar Surface Area (Angstrom squared)
Cyclosporine	0.5-4.0 [61]	1202.6	$C_{62}H_{111}N_{11}O_{12}$	3.96	4.09	4.09	11.83	279
Everolimus	~ 26 [142, 143]	958.2	$C_{53}H_{83}NO_{14}$	3.35	4.24	4.24	9.96	205
Mycophenolic acid	1-2.5 [4]	320.3	$C_{17}H_{20}O_6$	2.92	2.57	0.76	3.57	93
Prednisolone	6.3-27 [67, 144]	360.4	$C_{21}H_{28}O_5$	1.5	1.66	1.66	12.58	95
Sirolimus	~ 8 [145]	914.2	$C_{51}H_{79}NO_{13}$	3.54	4.21	4.21	9.96	195
Tacrolimus	1.0 [3]	804.0	$C_{44}H_{69}NO_{12}$	3.96	4.09	4.09	9.96	178

¥ pK value for the most acidic functional group.
*Information was obtained from ChemSpider (http://www.chemspider.com)
§Information was obtained from DrugBank (http://www.drugbank.ca)

Table 1-2. Published LC-MS/MS assays for quantification of immunosuppressive drugs in oral fluids (OF)

Drug	Patients	Collection device	Extraction method	Correlation	Chromatographic condition	Ion adducts Precursor ion (m/z) >product ion (m/z)	Ref.
CsA	RTRs (n = 15)	Passive drool	LLE with 94:6 vol/vol ACN: H_2O followed by SPE. R: 84.7% IV: 50 µL	r = 0.695 (P = 0.006)	Isocratic: 97:3 MeOH: 30 mmol/L ammonium acetate Column: Aqua Perfect C18 (MZ Analysentechnik) Run time: 5 min LLOQ: 1 µg/mL	[M + NH4]+. CsA: 1219.9>1202.9 IS: CsC	[39]
MPA	RTRs (n = 11)	Passive drool	LLE with two folds ACN/ dried and reconstituted with 85:15%: MeOH: 0.05% FA in H2O. R = >90%. IV: 20 µL	Total plasma MPA r = 0.91. Free plasma MPA r = 0.909	Gradient: 0.05% FA (A), MeOH (B) Column: Zorbax Rx C8 (Agilent Tech.) Run time: 7.5 min LLOQ: 2.5 µg/mL	[M + H] MPA: 319.0>190.8 IS: Indomethacin:	[38]
MPA	RTR (n = 9) HV (n = 8)	NR	LLE with equal volume ACN R: 84.1-86.7% IV: 10 µL	Total plasma MPA r = 0.838. Free plasma MPA r = 0.816	Isocratic: 45:55 (v: v) H_2O: ACN/0.1% FA Column: Allure PFP Propyl (RESTEK) Run time: 5 min LLOQ: 0.1 µg/mL.	$[M + H]^+$ MPA: 321.2>207.1 IS: Sulfadimethoxy-pyrimidine	[25]
MPA and MPAG	HV (n = 6)	Salivette polyethylene swab	LLE with two folds ACN/ dried and reconstituted with mobile phase R: MPA: 82.1%, MPAG: 65.7% IV: 5 µL	NR	Isocratic: 50% ACN/0.1% FA Column: Hypersil Gold C18 (Thermo Scientific) Run time: 2 min LLOQ: 5 µg/L.	$[M + H]^+$ MPA: 321.14>207.11 $[M+Na]^+$; MPAG: 519.29>343.24 IS: labeled MPA	[37]
TAC	RTRs (n = 37), children	Salivette polyester swab/ Passive drool	LLE with two fold ACN. R:NR. RV: 20 µL	$r^2 = 0.36$	Gradient: H_2O (A), MeOH (B) both contain 2 mM ammonium acetate/ 0.1 FA Column: C18 cartridge Run time: NR LLOQ: NR	NR	[24]

Abbreviations: Ammonium adduct: [M+NH4]+; Cyclosporine A: CsA; Cyclosporine C: CsC; Formic acid: FA; Healthy volunteers: HV; Injection volume: IV; Ion adduct [M+H]+; Lower limit of quantification: LLOQ; Methanol: MeOH; Mycophenolic acid: MPA; Mycophenolic acid glucuronide; Not reported: NR; Recovery: R; Renal transplant recipients: RTRs, Solid-phase extraction: SPE; Water: H2O.

Table 1-3. Published LC-MS/MS assays for quantification of immunosuppressive drugs in dried blood spot samples

Drug	Subj.	Blood volume/ drying time	Card type/ circle size/ punch size	Extraction method	Stability	Chromatographic Conditions	Adducts/ Precursor ion (m/z) > product ion (m/z)	Ref.
CsA	NR	50µL pipetted Venous blood/ dried overnight/ RT	Whatman 903/ 8mm	50% MeOH. Shaken at RT/ sonication IV: 20µL R: 97%.	17 days at RT 45 days at 4C°	Gradient: H2O (A), MeOH (B) both contain 2 mM ammonium acetate/ 0.1 FA Column: Xbridge™ RP18 (Waters) Run time: 4 mins. LLOQ: 25µg/mL	[M+NH4]+ CsA: 1219> 1202. IS: CsD	[79]
CsA	RTRs (n=42), renal& PTRs (n=2), LTRs (n=11)	A drop from collection devise/dried for 2hrs	Whatman 903/ 4mm	MeOH. Stirring IV: 35 µL R: NR	12 hrs at 8-20 °C	Isocratic: 97% MeOH contains 10 mM ammonium acetate and 0.1% acetic acid Column: Phenyl-Hexyl-RP (Phenomenex). Run time: 3 mins LLOQ: 4.5µg/L	[M+NH4]+ CsA: 1220>1203 IS: CsD	[15]

CsA, EVE, SIR, and TAC	NR	50µL, dry at RT/ overnight	Whatman 903/ 8mm	1:1 MeOH: Ethanol mixture. sonication/ dry at RT and reconstituted in mobile phase. IV: 50µL; R: CsA: 59.1-65.9%; EVE: 64.1-64.2%; SIR: 65.8-64.1%; TAC: 78.6-80.0%	5 months at 2-8C° for all analytes	Isocratic: 82% MeOH contains trifluoroacetic acid 0.05 % and 5 mM ammonium format Column: Symmetry Shield RP18 (Waters). Run time: 4.2 mins LLOQ: 23.6, 1.26, 1.34, and 1.14 µg/L for CsA, EVE, SIR, and TAC, respectively.	$[M+NH4]^+$ CsA: 1.219.7>1,202; EVE: 975.6>908.5 SIR: 931.5>864.4 TAC: 821.4>768.4 IS: ASC, CsD	[73]
CsA, EVE, SIR, and, TAC	HTRs, RTRs, LgTRs, LTRs, PTRs and small intestine (n=NR)	50µL pipetted	Whatman (31 ET CHR and FTA DMPK-C)/ 8mm	MeOH: H_2O (80:20 v/v%) mixture. Vortexed, soincated and stored at −20 for 10 mins IV: 20 µL; recovery: CsA: 89.1-121.3; EVE: 85.7-98.8; SIR: 88.7-92.4; TAC: 95.2-95.4	All analytes stable for at least 7 days at $22C^0$	Gradient: 20 mM ammonium formate buffer pH 3.5 (A), MeOH (B) Column: HyPURITYs C18 (ThermoFisher Scientific) Run time: 3.1 mins. LLOQ: 20, 1,1 and 1µg/L for CsA, EVE, SIR, and TAC, respectively.	M+NH4]+ CsA: 1.219.7>1,202; EVE: 975.6>908.5 SIR: 931.5>864.4 TAC: 821.4>768.4 IS: ASC, CsD	[87]

CsA and TAC	RTRs, HTRs, and LTRs (n=115)	50µL dried at RT/ 3hr.	Whatman 903/ 8 mm	0.01 mol/L ZnSO4. IV: 20µL R: NR.	CsA & TAC, 5days at 60 °C. SIR stable was for 5 days at 37C°	Gradient: H2O (A), B 100% MeOH, both contain 2 mM ammonium acetate and 0.1% FA. Column: Supelcosil LC-18-DB (Sigma-Aldrich). Run time: 3.5 mins LLOQ: 31, 1.2, and 1.2 µg/L for CsA, SIR, and TAC, respectively.	[M+NH4]+ CsA: 1219.9 > 1202.9 SIR: 931.6 > 864; TAC: 821.5 > 768.5 IS: ASC, CsD and labeled SIR	[77]
CsA and TAC	(n=150) / NR	25µL pipetted on the card/ dry at RT/ 3hrs.	Whatman 903/ 6mm	1:1 MeOH: ACN mixture, and sonication. IV: 10µL R: CsA: 92.8%; TAC: 95.5%.	14 days at RT	Gradient: H2O (A), MeOH (B) both contain 2mM ammonium acetate and 0.1% FA. Column: Acquity UPLC BEH C18 (Waters) Run time: 3 mins LLOQ: 75 µg/L and 2.5 µg/L for CsA, and TAC, respectively.	[M+NH4]+ CsA: 1220>1203 [M+NH4]+ CsA: 1220 > 1203 TAC: 821.6 >768.5 IS: ASC and labeled CsA	[74]

EVE	RTRs (n=11)	30µL on the card/ dry at RT/ 4hrs	Whatman 903/ 8mm/ 7.5 mm	Impregnated filter paper. The disc extracted with 1:10, MeOH: mobile phase (B). Sonicated IV: 50µL R: 76.5%.	3 days at 60 °C and 32 days at $4C^0$	Gradient: H2O (A), MeOH (B) both contain 2 mM ammonium acetate / 0.1% FA. Online SPE: Oasis HLB cartridge (Waters) Column: Atlantis dC18 (Waters) Run time: 6:30 mins LLOQ: 2µg/L	[M+NH4]+ EVE: 975.8 > 908.8 IS: 32-desmethoxy-rapamycin	[78]
MPA, MPAG	HV (n=NR)	20µL on the card; dry at RT/ 2hrs	Ahlstrom 226 card/ 3mm	1:1 H2O: IS mixture (ethanol containing 1% FA). Sonication. IV: 3µl R: MPA: 68.2-76.4; MPAG: 66.9-70.5	1 week at RT	Gradient: 20% ACN (A), 95 % ACN (B) both contain 0.1% FA. Online SPE: Hypersil Gold C18 (Thermo) Column: Atlantis T3 (Waters) Run time: 2mins LLOQ: 0.1µg/mL	[M + H]+. MPA: 321 > 207 MPAG: 514 > 207 IS: labeled MPA and MPAG	[88]
TAC	RTRs (n= 26, 26, 36)	30µL on the card; dried RT/ overnight	Whatman Schleicher & Schuell/ 8mm/7.5 mm	1:10 of MeOH: MeOH/ ACN (40:10 v/v), shake for 60 min. IV: 50µL R: 78%	1 day at 70 °C; 7 days at 37°C; 9 days at RT; 7 days at -20 °C	Gradient: H_2O (A), MeOH (B) both contain 2mM ammonium acetate and 0.1% FA. Online SPE: Oasis HLB cartridge (Waters) Column: Atlantis dC18 (Waters) Run time: 6.5mins LLOQ: 1µg/L	M+NH4]+ TAC: 821.4>768.3 IS: ASC	[84], [75], [81]

TAC	RTRs (n=21)	30–50µl from fingertip, dried 12h/ desiccator at RT	Whatman FTA DMPKA/ 6mm	MeOH/ ACN (80:20, v:v) mixture. Vortexed for 60 min/ SPE. Dried and reconstituted with ACN. IV: 10µL R: NR	One month when stored desiccated at RT	Gradient: H_2O (A), MeOH (B) both contain 2mM ammonium acetate and 0.1% FA Column: Nova-Pak (Waters) Run time: 2.4mins LLOQ: 1µg/L	[M+NH4]+ TAC 821> 768.2/786.2 IS: ASC	[76]
TAC		Pipette 30µL	Whatman 903/ 6mm	MeOH: H2O (10:90, v/v) sonication for 30 min. Add HCL (0.05 mol/L), and methyl tert-butyl ether. Organic phase dried/ reconstituted with 50% MeOH IV: NR; R: 76.6%	10 days at RT	Isocratic: 95% MeOH contains 2 mmol/L ammonium acetate / 0.1% FA (v/v) Column: XBridge Phenyl (Waters) Run time: 5mins LLOQ: 1µg/L.	[M+NH4]+ TAC 821.5> 768.4 IS: ASC	[143]

Abbreviations: Ammonium adduct: [M+NH4]+; Ascomycin: ASC; Cyclosporine A: CsA; Cyclosporine C: CsC; Cyclosporine D: CsD; Everolimus: EVE; Formic acid: FA; Healthy volunteers: HV; Heart transplant recipients: HTRs; Injection volume: IV; Ion adduct [M+H]+; Liver transplant recipients: LTRs; Lower limit of quantification: LLOQ; Lung transplant recipients: LgTRs; Methanol: MeOH; Mycophenolic acid: MPA; Mycophenolic acid glucuronide; Not report: NR; Recovery: R; Renal transplant recipients: RTRs; Room temperature: RT; Sirolimus: SIR; Solid phase extraction: SPE; Tacrolimus : TAC; Waters :H_2O.

Table 1-4. Published LC-MS/MS assays for quantification of immunosuppressive drugs in fingerprick samples

Drug/ Reference	Subjects	Blood sample volume	Extraction method	Chromatographic conditions	Ion adducts Precursor ion (m/z) > product ion (m/z)	REF
CsA	HTRs and LgTRs (n=65); RTRs (n=33)	10 µL	Pretreatment with 0.1 mmol zinc sulfate solution and LLE with ACN. IV: 5 µL.	Gradient: H_2O (A), MeOH (B), both contain 2 mmol ammonium acetate/ 0.1 FA Online SFE: SecurityGuard C18 cartridge (Phenomenex) Run time: 2.5 min LLOQ: 10 µg/L	$[M + NH4]^+$ CsA 1,220>1,203 IS: ASC /CsD	[11-13]
TAC	RTRs (n=33); RTRs & pancreas (n=2); and RTRs & HTRs (n=1) children	10 µL	Pretreatment with 0.1 mmol zinc sulfate solution and LLE with ACN. IV: 10 µL	Gradient: H_2O (A), MeOH (B), both containing 2 mmol ammonium acetate/ 0.1 FA Online SFE: SecurityGuard C18 cartridge (Phenomenex) Run time: NR LLOQ: 0.5 µg/L	$[M + NH4]^+$ TAC: 821>768 IS: ASC	[14]

Abbreviations: Ammonium adduct: [M+NH4]+; Ascomycin: ASC; Cyclosporine A: CsA; Cyclosporine D: CsD; Formic acid: FA; Heart transplant recipients: HTRs; Injection volume: IV; Lower limit of quantification: LLOQ; Lung transplant recipients: LgTRs; Methanol: MeOH; Recovery: R; Renal transplant recipients: RTRs, Solid-phase extraction: SPE; Tacrolimus: TAC; Water:H_2O.

Table 1-5. LC-MS/MS published assays for quantification of immunosuppressive drugs in lymphocytes

Drug	Subj.	Extraction	Chromatographic conditions	Ion adducts: Precursor ion (m/z) > product ion (m/z).	Ref.
CsA	HV (n=NR); LTRs, LgTRs, and RTRs (n=64)	At 4 °C to prevent efflux of CsA. Lymphocyte isolated from 8 mL blood with BD Vacutainer® CPT™ Cell Preparation Tubes Cells lysed with MeOH/dry reconstituted with MeOH IV: 40 µL R: 98%	Gradient: H2O (A), MeOH (B) Online SPE: XTerra™ MS C8 Column: XTerra™ MS C18 (Waters) Run time: 31 min LLOQ: 5 ng/mL; 0.5 fg/PBMC	LC/MS. $[M+Na]^+$ CsA: 1224.7/NR IS: 27 demethoxy-sirolimus	[114] [105] [104]
CsA	Early RTRs (n=20); HTRs (n=10)	Verapamil added prevent efflux of CsA. T-lymphocyte isolated from 7 mL blood with Prepacyte®. Cells were lysed and protein precipitated with MeOH: ACN: water (1:1:3) followed by SPE. IV: 100 µL R: CsA and metabolites 73.5- 98.6	Gradient: ACN/20 mM ammonium formate buffer pH 3.6 (20:80) (A), ACN/ (NH4+COO−) pH 3.6 (80:20) (B) SPE: Water Oasis®, HLB 1 cc, 30 mg Column: C8, (Thermo Electron Corp) Run time: 38 min LLOQ: 0.25 ng/mL	$[M+H]^+$. CsA: 1203.7>1101.7/1185.7 IS: CsC	[115] [112]
CsA and metabolites	Early (n = 57) and chronic RTRs (n=54)	Verapamil added to prevent efflux of CsA. Lymphocyte isolated from 1.5 mL blood with Histopaque 1077 solution. Precipitation solution ACN: MeOH (40:60, v/v); (v:v:v) IV: NR, R: CsA and metabolites 71.9-78.4%.	Gradient: 5% ACN (A), 95% ACN (B); both contain 2 mM/L ammonium acetate/ 0.1 FA Column: Acquity UPLC RP BEH, C18 Run time: 5 min LLOQ: 5 ng/mL	$[M+H]^+$. CsA: 1202.8>156.2. IS: CsD	[116]

CsA	HTRs (n=17)	10 mL whole blood incubated with E-Rosette solution, followed by density separation using Histiopaque solution. IV: 1 µL R: NR.	Isocratic: ACN: 2 mM ammonium acetate: FA (70:30:0.1) Column: Supelco LC-CN Run time: NA LLOQ: 1 pg.	$[M+NH]^+$. CsA: 1,220.8>1,202.7 IS: CsD	[119]
EVE	HTRs (n=36)	At 4 °C to prevent efflux of EVE Lymphocyte isolated from 8 mL blood with BD Vacutainer® CPT™ Cell Preparation Tubes. Cells were lysed with MeOH dry & reconstituted with MeOH. Extract one part with 4:5:3 parts of zinc sulfate 0.1 M: 5: H2O: ACN. IV: 20 µL R: 79.4-87.1%.	Gradient: H2O (A), MeOH (B); both contain 2 mM ammonium acetate/ 0.1% FA Column: MassTrak TDM C18 (Waters). Run time: 1.5 min LLOQ: 1.25 ng/mL	$[M+NH4]^+$ EVE: m/z 975.5> 908.5 IS: ASC	[126]
TAC	RTRs (n=65)	At 4 °C to prevent efflux of TAC Lymphocyte isolated from 7 mL blood with Ficoll-Paque Plus solution. Reconstituted with PBS and extracted with 1-chlorobutan. Organic phase dried and reconstituted with MeOH containing 2 mmol/L ammonium acetate / 0.1% FA IV: 25 µL R: 74.8% to 86.7%	Isocratic: 90% ACN contains 2 mM ammonium acetate/ 0.1% FA Column: Xterra C18 (Waters) Run time: NR LLOQ: 0.01 ng/mL/0.006 ng/ 106 PBMCs	$[M+NH4]^+$ TAC: m/z 821.6>768.5 IS: ASC	[117,118]
TAC	HTRs (n=24)	At 4 °C to prevent efflux of TAC. Lymphocyte isolated from 7 mL blood with BD Vacutainer® CPT™ Cell Preparation Tubes. Cells lysed with MeOH dried/ reconstituted with MeOH and extracted with zinc sulfate 0.1 M: 1: 2.5 (v:v) IV: 20 µL R: 97.2-103.4%.	Gradient: H2O (A); MeOH (B) both contain 2 mM ammonium acetate/ 0.1% FA Column: MassTrak TDM C18 (Waters) Run time: NR LLOQ: 12.5 pg/million PBMCs	NR	[113]

Abbreviations: Ammonium adduct: [M+NH4]+; Ascomycin: ASC; Cyclosporine A: CsA; Cyclosporine C: CsC; Cyclosporine D: CsD; Everolimus: EVE; Formic acid: FA; Heart transplant recipients: HTRs; Injection volume: IV; Ion adduct [M+H]+; Liver transplant recipients: LTRs; Lower limit of quantification: LLOQ; Lung transplant recipients: LgTRs; Methanol: MeOH; Not reported: NR; Phosphate buffer solution: PBS; Recovery: R; Renal transplant recipients: RTRs, Solid-phase extraction: SPE; Tacrolimus: TAC.

Table 1-6. LC-MS/MS published assays for quantification of immunosuppressive drugs in biopsies from transplanted organs

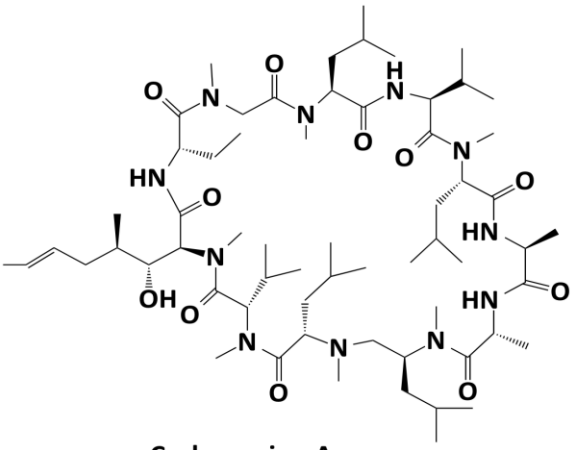
Drug	Subj.	Extraction/ Injection volume/ Recovery	Correl-ation Blood vs. Tissue	Chromatographic conditions	Ion adducts: Precursor ion (m/z) > product ion (m/z).	Ref.
cyclosporine	RTRs (n=21)	Tissues solubilized in digestion buffer (Proteinase K solution in ATL buffer) at 56 °C/ 2 hr. Precipitate with 0.4 M zinc sulfate: MeOH, 20:80 (v:v) IV: 25 µL R: NR	r = 0.168, P = 0.53	Gradient: (A): 50% MeOH, MeOH (B), both contain 2 mM ammonium acetate/ 0.1% FA On line cleaning: POROS R1/20 Column: Luna Phenyl-Hexyl (Phenomenex) Run time: 5.5 min LLOQ: 1 ng/mL	[M + NH4]+ CsA: 1220.0>1203.0 IS: labeled CsA	[137]
cyclosporine	Biopsies (n=19) from 7 HTRs	Tissues homogenized with water and mixed with two parts of ACN:MeOH: H_2O (1:1:3, v:v:v). Precipitation with ACN. The organic phase was dried and reconstituted with MPs A:B, 65:35 (v:v) IV: 20 µL R: NR	NR	Gradient: 20% ACN (A), 80% ACN (B), both contain 20 mM ammonium format Column: (Acquity) UPLC C18 (Waters) Run time: 36 min LLOQ: 0.25 ng/mL	[M+H]+ CsA: 1203.7>1101.7/ 1185.7 IS: CsC	[112]
MPA	Biopsies (n=4) from 4 RTRs	Tissues grounded to a fine powder and reconstituted with PBS (pH 7.4). Add 60 µL of HCl (0.4 M) and 1 mL of tertiary-butyl methyl ether. Evaporate/ reconstitute with 1:1 (v:v) MeOH: H2O. IV: NR	NR	Gradient: H2O (A), and MeOH (B), both containing 2 mM ammonium acetate / 0.1% formic acid Column: Luna Phenyl-Hexyl (Phenomenex) Run time: 2.2 min LLOQ: 0.6 ng/mL	[M + H]+. MPA: 321.1>207.3 IS: N-phthaloyl-l-phenylalanine	[139]

		R: 97%				
TAC	Biopsies (NR) from 90 and 146 LTRs	Tissues homogenized PBS (0.1 mol/L, pH = 6.5. Extracted with MeOH /ethyl acetate, dried and reconstituted with MeOH. IV: 20 µL R: 75.3 -83.1%	Banff score (r^2 = 0.984, P = 0.002)	Isocratic: 70% MeOH (B) containing 2 mM ammonium acetate/ 0.1% FA Column: C18 cartridge, (Phenomenex) Run time: 1.5 min LLOQ: 5 pg/mg	[M + NH4]+ TAC: 822 > 768 IS: ASC	[136] [117]
TAC	Biopsies (n=6) from 2 RTRs	Tissues solubilized in digestion buffer. Mix with 7 µL of IS + 300 µL water+1 mL of tert-butyl methyl ether in glass tube. Organic phase evaporated and reconstituted in 50:50 MeOH: H2O IV: 25 µL R: 70%	NR	Gradient: H2O (A) and MeOH (B) both contain 2 mM ammonium acetate/ 0.1% FA Column: Luna Phenyl-Hexyl (Phenomenex) Run time: 2 min LLOQ: 0.04 ng/mL	[M + NH4]+ TAC: 821.5>768.6 IS: ASC	[138]

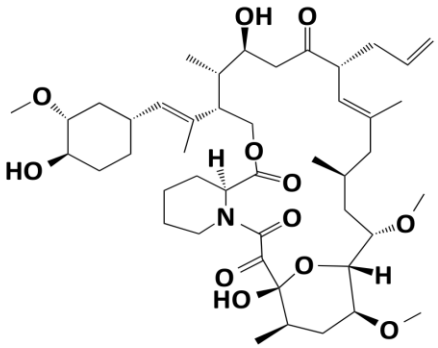
Abbreviations: Ammonium adduct: [M+NH4]+; Ascomycin: ASC; Cyclosporine A: CsA; Cyclosporine C: CsC; Everolimus: EVE; Formic acid: FA; Heart transplant recipients: HTRs; Injection volume: IV; Ion adduct [M+H]+; Liver transplant recipients: LTRs; Lower limit of quantification: LLOQ; Methanol: MeOH; Mycophenolic acid: MPA; Not reported: NR; Phosphate buffer solution: PBS; Recovery: R; Renal transplant recipients: RTRs, Solid-phase extraction: SPE; Tacrolimus: TAC.

Figures

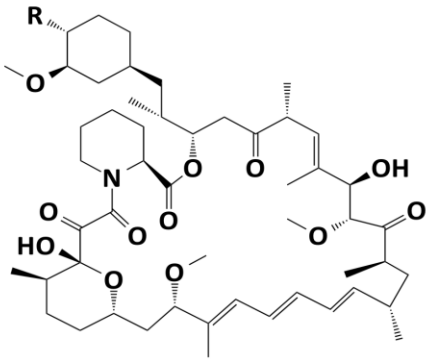
Figure 1-1, Chemical structure of immunosuppressive agents included in this review



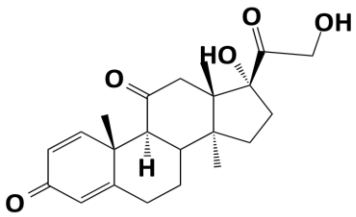
Cyclosporine A



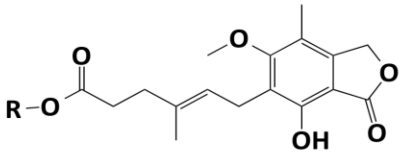
Tacrolimus



Sirolimus R = OH
Everolimus R = OCH3OH



Prednisolon



Mcophenolic acid R = H

Mycophenolic acid glucrounide: R =

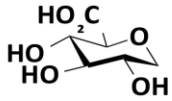
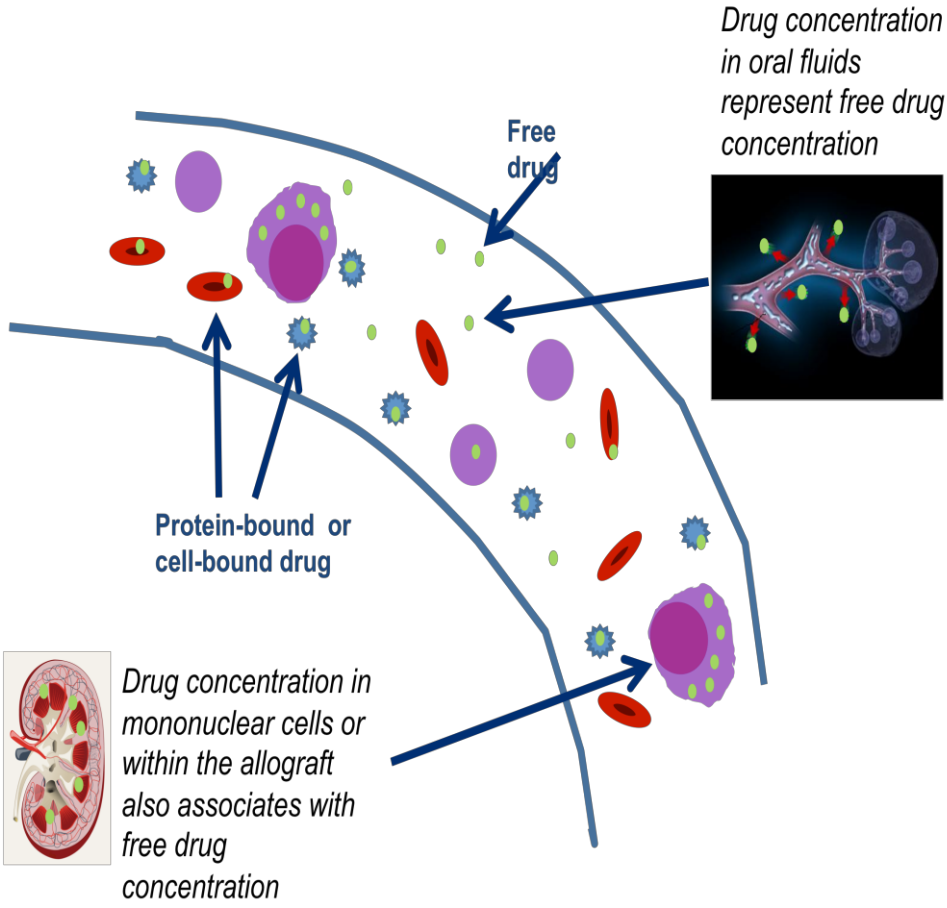


Figure 1-2. Schematic diagram depicting the relationship between bound and unbound concentration of an immunosuppressive agent with the concentration at allograft or peripheral blood mononuclear cells as well as concentration in oral fluids or saliva.



References

1. Schiff J, Cole E, Cantarovich M. Therapeutic monitoring of calcineurin inhibitors for the nephrologist. *Clinical journal of the American Society of Nephrology : CJASN*, 2(2), 374-384 (2007).

2. Johnston A, Holt DW. Therapeutic drug monitoring of immunosuppressant drugs. *British journal of clinical pharmacology*, 47(4), 339-350 (1999).

3. Staatz CE, Tett SE. Clinical pharmacokinetics and pharmacodynamics of tacrolimus in solid organ transplantation. *Clinical pharmacokinetics*, 43(10), 623-653 (2004).

4. Staatz CE, Tett SE. Clinical pharmacokinetics and pharmacodynamics of mycophenolate in solid organ transplant recipients. *Clinical pharmacokinetics*, 46(1), 13-58 (2007).

5. Mohammadpour N, Elyasi S, Vahdati N, Mohammadpour AH, Shamsara J. A review on therapeutic drug monitoring of immunosuppressant drugs. *Iranian journal of basic medical sciences*, 14(6), 485-498 (2011).

6. Korecka M, Shaw LM. Review of the newest HPLC methods with mass spectrometry detection for determination of immunosuppressive drugs in clinical practice. *Annals of transplantation : quarterly of the Polish Transplantation Society*, 14(2), 61-72 (2009).

7. Sallustio BC. LC-MS/MS for immunosuppressant therapeutic drug monitoring. *Bioanalysis*, 2(6), 1141-1153 (2010).

8. Vogeser M, Kirchhoff F. Progress in automation of LC-MS in laboratory medicine. *Clinical biochemistry*, 44(1), 4-13 (2011).

9. Marinova M, Artusi C, Brugnolo L, Antonelli G, Zaninotto M, Plebani M. Immunosuppressant therapeutic drug monitoring by LC-MS/MS: workflow optimization through automated processing of whole blood samples. *Clinical biochemistry*, 46(16-17), 1723-1727 (2013).

10. Spooner N, Lad R, Barfield M. Dried blood spots as a sample collection technique for the determination of pharmacokinetics in clinical studies: considerations for the validation of a quantitative bioanalytical method. *Anal. Chem.*, 81(4), 1557-1563 (2009).

11. Webb NJ, Coulthard MG, Trompeter RS *et al*. Correlation between finger-prick and venous ciclosporin levels: association with gingival overgrowth and hypertrichosis. *Pediatric nephrology (Berlin, Germany)*, 22(12), 2111-2118 (2007).

12. Yonan N, Martyszczuk R, Machaal A, Baynes A, Keevil BG. Monitoring of cyclosporine levels in transplant recipients using self-administered fingerprick sampling. *Clinical transplantation*, 20(2), 221-225 (2006).

13. Keevil BG, Tierney DP, Cooper DP, Morris MR, Machaal A, Yonan N. Simultaneous and rapid analysis of cyclosporin A and creatinine in finger prick blood samples using liquid chromatography tandem mass spectrometry and its application in C2 monitoring. *Therapeutic drug monitoring*, 24(6), 757-767 (2002).

14. Webb NJ, Roberts D, Preziosi R, Keevil BG. Fingerprick blood samples can be used to accurately measure tacrolimus levels by tandem mass spectrometry. *Pediatric transplantation*, 9(6), 729-733 (2005).

15. Leichtle AB, Ceglarek U, Witzigmann H, Gabel G, Thiery J, Fiedler GM. Potential of dried blood self-sampling for cyclosporine c(2) monitoring in transplant outpatients. *Journal of transplantation*, 2010, 201918 (2010).

16. Murthy JN, Yatscoff RW, Soldin SJ. Cyclosporine metabolite cross-reactivity in different cyclosporine assays. *Clinical biochemistry*, 31(3), 159-163 (1998).

17. Murthy JN, Davis DL, Yatscoff RW, Soldin SJ. Tacrolimus metabolite cross-reactivity in different tacrolimus assays. *Clinical biochemistry*, 31(8), 613-617 (1998).

18. Dietemann J, Berthoux P, Gay-Montchamp JP, Batie M, Berthoux F. Comparison of ELISA method versus MEIA method for daily practice in the therapeutic monitoring of tacrolimus. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*, 16(11), 2246-2249 (2001).

19. Napoli KL. Is microparticle enzyme-linked immunoassay (MEIA) reliable for use in tacrolimus TDM? Comparison of MEIA to liquid chromatography with mass spectrometric detection using longitudinal trough samples from transplant recipients. *Therapeutic drug monitoring*, 28(4), 491-504 (2006).

20. Moes DJ, Press RR, de Fijter JW, Guchelaar HJ, den Hartigh J. Liquid chromatography-tandem mass spectrometry outperforms fluorescence polarization immunoassay in monitoring everolimus therapy in renal transplantation. *Therapeutic drug monitoring*, 32(4), 413-419 (2010).

21. Buchwald A, Winkler K, Epting T. Validation of an LC-MS/MS method to determine five immunosuppressants with deuterated internal standards including MPA. *BMC clinical pharmacology*, 12, 2 (2012).

22. Zimmer D. Introduction to Quantitative Liquid Chromatography-Tandem Mass Spectrometry. *Chromatographia*, 57(SUPL.), S-325-S-332 (2003).

23. Holcapek M, Kolarova L, Nobilis M. High-performance liquid chromatography-tandem mass spectrometry in the identification and determination of phase I and phase II drug metabolites. *Analytical and bioanalytical chemistry*, 391(1), 59-78 (2008).

24. Belostotsky V, Adaway J, Keevil BG, Cohen DR, Webb NJ. Measurement of saliva tacrolimus levels in pediatric renal transplant recipients. *Pediatric nephrology (Berlin, Germany)*, 26(1), 133-138 (2011).

25. Shen B, Li S, Zhang Y *et al*. Determination of total, free and saliva mycophenolic acid with a LC-MS/MS method: application to pharmacokinetic study in healthy volunteers and renal transplant patients. *Journal of pharmaceutical and biomedical analysis*, 50(3), 515-521 (2009).

26. Dams R, Huestis MA, Lambert WE, Murphy CM. Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: influence of ionization type, sample preparation, and biofluid. *J*. *Am*. *Soc*. *Mass Spectrom*., 14(11), 1290-1294 (2003).

27. Mei H, Hsieh Y, Nardo C *et al*. Investigation of matrix effects in bioanalytical high-performance liquid chromatography/tandem mass spectrometric assays: application to drug discovery. *Rapid communications in mass spectrometry : RCM*, 17(1), 97-103 (2003).

28. Chambers E, Wagrowski-Diehl DM, Lu Z, Mazzeo JR. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *Journal of chromatography*. *B, Analytical technologies in the biomedical and life sciences*, 852(1-2), 22-34 (2007).

29. Feller K, le Petit G. On the distribution of drugs in saliva and blood plasma. *International journal of clinical pharmacology and biopharmacy*, 15(10), 468-469 (1977).

30. Horning MG, Brown L, Nowlin J, Lertratanangkoon K, Kellaway P, Zion TE. Use of saliva in therapeutic drug monitoring. *Clinical chemistry*, 23(2 PT. 1), 157-164 (1977).

31. Mucklow JC, Bending MR, Kahn GC, Dollery CT. Drug concentration in saliva. *Clinical pharmacology and therapeutics*, 24(5), 563-570 (1978).

32. Drobitch RK, Svensson CK. Therapeutic drug monitoring in saliva. An update. *Clinical pharmacokinetics*, 23(5), 365-379 (1992).

33. Gorodischer R, Koren G. Salivary excretion of drugs in children: theoretical and practical issues in therapeutic drug monitoring. *Developmental pharmacology and therapeutics*, 19(4), 161-177 (1992).

34. Haeckel R. Factors influencing the saliva/plasma ratio of drugs. *Ann. N. Y. Acad. Sci.*, 694, 128-142 (1993).

35. Jusko WJ, Milsap RL. Pharmacokinetic principles of drug distribution in saliva. *Ann. N. Y. Acad. Sci.*, 694, 36-47 (1993).

36. Coates JE, Lam SF, McGaw WT. Radioimmunoassay of salivary cyclosporine with use of 125I-labeled cyclosporine. *Clinical chemistry*, 34(8), 1545-1551 (1988).

37. Wiesen MH, Farowski F, Feldkotter M, Hoppe B, Muller C. Liquid chromatography-tandem mass spectrometry method for the quantification of mycophenolic acid and its phenolic glucuronide in saliva and plasma using a standardized saliva collection device. *Journal of chromatography. A*, 1241, 52-59 (2012).

38. Mendonza AE, Gohh RY, Akhlaghi F. Analysis of mycophenolic acid in saliva using liquid chromatography tandem mass spectrometry. *Therapeutic drug monitoring*, 28(3), 402-406 (2006).

39. Mendonza A, Gohh R, Akhlaghi F. Determination of cyclosporine in saliva using liquid chromatography-tandem mass spectrometry. *Therapeutic drug monitoring*, 26(5), 569-575 (2004).

40. Swallow V, Hughes J, Roberts D, Webb NJ. Assessing children's and parents' opinions on salivary sampling for therapeutic drug monitoring. *Nurse Res.*, 19(3), 32-37 (2012).

41. Tennison M, Ali I, Miles MV, D'Cruz O, Vaughn B, Greenwood R. Feasibility and acceptance of salivary monitoring of antiepileptic drugs via the US Postal Service. *Therapeutic drug monitoring*, 26(3), 295-299 (2004).

42. Gorodischer R, Burtin P, Hwang P, Levine M, Koren G. Saliva versus blood sampling for therapeutic drug monitoring in children: patient and parental preferences and an economic analysis. *Therapeutic drug monitoring*, 16(5), 437-443 (1994).

43. Zahir H, McCaughan G, Gleeson M, Nand RA, McLachlan AJ. Changes in tacrolimus distribution in blood and plasma protein binding following liver transplantation. *Therapeutic drug monitoring*, 26(5), 506-515 (2004).

44. Akhlaghi F, Ashley J, Keogh A, Brown K. Cyclosporine plasma unbound fraction in heart and lung transplantation recipients. *Therapeutic drug monitoring*, 21(1), 8-16 (1999).

45. Haeckel R, Hanecke P. Application of saliva for drug monitoring. An in vivo model for transmembrane transport. *European journal of clinical chemistry and clinical biochemistry : journal of the Forum of European Clinical Chemistry Societies*, 34(3), 171-191 (1996).

46. Kaufman E, Lamster IB. The diagnostic applications of saliva--a review. *Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists*, 13(2), 197-212 (2002).

47. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced drug delivery reviews*, 46(1-3), 3-26 (2001).

48. Liu H, Delgado MR. Therapeutic drug concentration monitoring using saliva samples. Focus on anticonvulsants. *Clinical pharmacokinetics*, 36(6), 453-470 (1999).

49. Idkaidek NM. Interplay of biopharmaceutics, biopharmaceutics drug disposition and salivary excretion classification systems. *Saudi pharmaceutical journal : SPJ : the official publication of the Saudi Pharmaceutical Society*, 22(1), 79-81 (2014).

50. Idkaidek N, Arafat T. Saliva versus plasma pharmacokinetics: theory and application of a salivary excretion classification system. *Molecular pharmaceutics*, 9(8), 2358-2363 (2012).

51. Drummer OH. Introduction and review of collection techniques and applications of drug testing of oral fluid. *Therapeutic drug monitoring*, 30(2), 203-206 (2008).

52. Granger DA, Shirtcliff EA, Booth A, Kivlighan KT, Schwartz EB. The "trouble" with salivary testosterone. *Psychoneuroendocrinology*, 29(10), 1229-1240 (2004).

53. Groschl M, Kohler H, Topf HG, Rupprecht T, Rauh M. Evaluation of saliva collection devices for the analysis of steroids, peptides and therapeutic drugs. *Journal of pharmaceutical and biomedical analysis*, 47(3), 478-486 (2008).

54. Shaefer MS, Collier DS, Haven MC *et al*. Falsely elevated FK-506 levels caused by sampling through central venous catheters. *Transplantation*, 56(2), 475-476 (1993).

55. Blifeld C, Ettenger RB. Measurement of cyclosporine levels in samples obtained from peripheral sites and indwelling lines. *N. Engl. J. Med.*, 317(8), 509 (1987).
56. Lillsunde P. Analytical techniques for drug detection in oral fluid. *Therapeutic drug monitoring*, 30(2), 181-187 (2008).

57. Langel K, Engblom C, Pehrsson A, Gunnar T, Ariniemi K, Lillsunde P. Drug testing in oral fluid-evaluation of sample collection devices. *Journal of analytical toxicology*, 32(6), 393-401 (2008).

58. Kivlighan KT, Granger DA, Schwartz EB, Nelson V, Curran M, Shirtcliff EA. Quantifying blood leakage into the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva. *Hormones and behavior*, 46(1), 39-46 (2004).

59. Akhlaghi F, McLachlan AJ, Keogh AM, Brown KF. Effect of simvastatin on cyclosporine unbound fraction and apparent blood clearance in heart transplant recipients. *British journal of clinical pharmacology*, 44(6), 537-542 (1997).

60. Akhlaghi F, Trull AK. Distribution of cyclosporin in organ transplant recipients. *Clinical pharmacokinetics*, 41(9), 615-637 (2002).

61. Lindholm A, Henricsson S. Intra- and interindividual variability in the free fraction of cyclosporine in plasma in recipients of renal transplants. *Therapeutic drug monitoring*, 11(6), 623-630 (1989).

62. Akhlaghi F, Keogh AM, Brown KF. Unbound cyclosporine and allograft rejection after heart transplantation. *Transplantation*, 67(1), 54-59 (1999).

63. Zahir H, McCaughan G, Gleeson M, Nand RA, McLachlan AJ. Factors affecting variability in distribution of tacrolimus in liver transplant recipients. *British journal of clinical pharmacology*, 57(3), 298-309 (2004).

64. Vogeser M, Zachoval R, Spohrer U, Jacob K. Potential lack of specificity using electrospray tandem-mass spectrometry for the analysis of mycophenolic acid in serum. *Therapeutic drug monitoring*, 23(6), 722-724 (2001).

65. Spies CM, Strehl C, van der Goes MC, Bijlsma JW, Buttgereit F. Glucocorticoids. *Best practice & research. Clinical rheumatology*, 25(6), 891-900 (2011).

66. Bergmann TK, Barraclough KA, Lee KJ, Staatz CE. Clinical pharmacokinetics and pharmacodynamics of prednisolone and prednisone in solid organ transplantation. *Clinical pharmacokinetics*, 51(11), 711-741 (2012).

67. Ionita IA, Ogasawara K, Gohh RY, Akhlaghi F. Pharmacokinetics of total and unbound prednisone and prednisolone in stable kidney transplant recipients with diabetes mellitus. *Therapeutic drug monitoring*, 36(4), 448-455 (2014).

68. Meffin PJ, Brooks PM, Sallustio BC. Alterations in prednisolone disposition as a result of time of administration, gender and dose. *British journal of clinical pharmacology*, 17(4), 395-404 (1984).

69. Teeninga N, Guan Z, Freijer J *et al*. Monitoring prednisolone and prednisone in saliva: a population pharmacokinetic approach in healthy volunteers. *Therapeutic drug monitoring*, 35(4), 485-492 (2013).

70. Ruiter AF, Teeninga N, Nauta J, Endert E, Ackermans MT. Determination of unbound prednisolone, prednisone and cortisol in human serum and saliva by on-line solid-phase extraction liquid chromatography tandem mass spectrometry and potential implications for drug monitoring of prednisolone and prednisone in saliva. *Biomedical chromatography : BMC*, 26(7), 789-796 (2012).

71. den Burger JC, Wilhelm AJ, Chahbouni A, Vos RM, Sinjewel A, Swart EL. Analysis of cyclosporin A, tacrolimus, sirolimus, and everolimus in dried blood spot samples using liquid chromatography tandem mass spectrometry. *Analytical and bioanalytical chemistry*, 404(6-7), 1803-1811 (2012).

72. Hinchliffe E, Adaway JE, Keevil BG. Simultaneous measurement of cyclosporin A and tacrolimus from dried blood spots by ultra high performance liquid chromatography tandem mass spectrometry. *Journal of chromatography*. *B, Analytical technologies in the biomedical and life sciences*, 883-884, 102-107 (2012).

73. Hoogtanders K, van der Heijden J, Christiaans M, van de Plas A, van Hooff J, Stolk L. Dried blood spot measurement of tacrolimus is promising for patient monitoring. *Transplantation*, 83(2), 237-238 (2007).

74. Koop DR, Bleyle LA, Munar M, Cherala G, Al-Uzri A. Analysis of tacrolimus and creatinine from a single dried blood spot using liquid chromatography tandem mass spectrometry. *Journal of chromatography*. *B, Analytical technologies in the biomedical and life sciences*, 926, 54-61 (2013).

75. Sadilkova K, Busby B, Dickerson JA, Rutledge JC, Jack RM. Clinical validation and implementation of a multiplexed immunosuppressant assay in dried blood spots by LC-MS/MS. *Clinica chimica acta; international journal of clinical chemistry*, 421, 152-156 (2013).

76. van der Heijden J, de Beer Y, Hoogtanders K *et al*. Therapeutic drug monitoring of everolimus using the dried blood spot method in combination

with liquid chromatography-mass spectrometry. *Journal of pharmaceutical and biomedical analysis*, 50(4), 664-670 (2009).

77. Wilhelm AJ, den Burger JC, Vos RM, Chahbouni A, Sinjewel A. Analysis of cyclosporin A in dried blood spots using liquid chromatography tandem mass spectrometry. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 877(14-15), 1595-1598 (2009).

78. Lampe D, Scholz D, Prumke HJ, Blank W, Huller H. Capillary blood, dried on filter paper, as sample for monitoring cyclosporin A concentrations. *Clinical chemistry*, 33(9), 1643-1644 (1987).

79. Cheung CY, van der Heijden J, Hoogtanders K *et al*. Dried blood spot measurement: application in tacrolimus monitoring using limited sampling strategy and abbreviated AUC estimation. *Transplant international : official journal of the European Society for Organ Transplantation*, 21(2), 140-145 (2008).

80. Keevil BG. The analysis of dried blood spot samples using liquid chromatography tandem mass spectrometry. *Clinical biochemistry*, 44(1), 110-118 (2011).

81. Acott PD. Home fingerprick sampling for immunosuppressant drug monitoring in pediatric renal transplant patients. *Nature clinical practice. Nephrology*, 2(6), 304-305 (2006).

82. Hoogtanders K, van der Heijden J, Christiaans M, Edelbroek P, van Hooff JP, Stolk LM. Therapeutic drug monitoring of tacrolimus with the dried blood spot method. *Journal of pharmaceutical and biomedical analysis*, 44(3), 658-664 (2007).

83. Li Q, Cao D, Huang Y, Xu H, Yu C, Li Z. Development and validation of a sensitive LC-MS/MS method for determination of tacrolimus on dried blood spots. *Biomedical chromatography : BMC*, 27(3), 327-334 (2013).

84. Bowen CL, Dopson W, Kemp DC, Lewis M, Lad R, Overvold C. Investigations into the environmental conditions experienced during ambient sample transport: impact to dried blood spot sample shipments. *Bioanalysis*, 3(14), 1625-1633 (2011).

85. Holub M, Tuschl K, Ratschmann R *et al*. Influence of hematocrit and localisation of punch in dried blood spots on levels of amino acids and acylcarnitines measured by tandem mass spectrometry. *Clinica chimica acta; international journal of clinical chemistry*, 373(1-2), 27-31 (2006).

86. Koster RA, Alffenaar JW, Greijdanus B, Uges DR. Fast LC-MS/MS analysis of tacrolimus, sirolimus, everolimus and cyclosporin A in dried blood spots and the influence of the hematocrit and immunosuppressant concentration on recovery. *Talanta*, 115, 47-54 (2013).

87. Heinig K, Bucheli F, Hartenbach R, Gajate-Perez A. Determination of mycophenolic acid and its phenyl glucuronide in human plasma, ultrafiltrate, blood, DBS and dried plasma spots. *Bioanalysis*, 2(8), 1423-1435 (2010).

88. LifeSciences G. Grade 31ET Chr Cellulose Chromatography Papers. (LifeSciences, GH) http://www.gelifesciences.com/webapp/wcs/stores/servlet/ProductDisplay?categoryId=104361&catalogId=10101&productId=15116&storeId=11787&langId=-1

89. Li W, Tse FL. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. *Biomedical chromatography : BMC*, 24(1), 49-65 (2010).

90. PerkinElmer. PerkinElmer 226 Sample Collection Devices – Clinical. (PerkinElmer). http://www.perkinelmer.com/pages/060/newbornscreening/default.xhtml

91. Besarab A, Bolton WK, Browne JK *et al*. The effects of normal as compared with low hematocrit values in patients with cardiac disease who are receiving hemodialysis and epoetin. *The New England journal of medicine*, 339(9), 584-590 (1998).

92. Wilhelm AJ, den Burger JC, Chahbouni A, Vos RM, Sinjewel A. Analysis of mycophenolic acid in dried blood spots using reversed phase high performance liquid chromatography. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 877(30), 3916-3919 (2009).

93. Spooner N, Denniff P, Michielsen L *et al*. A device for dried blood microsampling in quantitative bioanalysis: overcoming the issues associated blood hematocrit. *Bioanalysis*, 1-7 (2014).

94. Denniff P, Spooner N. Volumetric absorptive microsampling: a dried sample collection technique for quantitative bioanalysis. *Analytical chemistry*, 86(16), 8489-8495 (2014).

95. Merton G, Jones K, Lee M, Johnston A, Holt DW. Accuracy of cyclosporin measurements made in capillary blood samples obtained by skin puncture. *Therapeutic drug monitoring*, 22(5), 594-598 (2000).

96. Wallemacq P, Armstrong VW, Brunet M *et al*. Opportunities to optimize tacrolimus therapy in solid organ transplantation: report of the European consensus conference. *Therapeutic drug monitoring*, 31(2), 139-152 (2009).

97. Batiuk TD, Pazderka F, Enns J, DeCastro L, Halloran PF. Cyclosporine inhibition of calcineurin activity in human leukocytes in vivo is rapidly reversible. *The Journal of clinical investigation*, 96(3), 1254-1260 (1995).

98. Thomson AW, Bonham CA, Zeevi A. Mode of action of tacrolimus (FK506): molecular and cellular mechanisms. *Therapeutic drug monitoring*, 17(6), 584-591 (1995).

99. Dumont FJ, Su Q. Mechanism of action of the immunosuppressant rapamycin. *Life Sci*., 58(5), 373-395 (1996).

100. Kemnitz J, Uysal A, Haverich A *et al*. Multidrug resistance in heart transplant patients: a preliminary communication on a possible mechanism of therapy-resistant rejection. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation*, 10(2), 201-210 (1991).

101. Albermann N, Schmitz-Winnenthal FH, Z'Graggen K *et al*. Expression of the drug transporters MDR1/ABCB1, MRP1/ABCC1, MRP2/ABCC2, BCRP/ABCG2, and PXR in peripheral blood mononuclear cells and their relationship with the expression in intestine and liver. *Biochem*. *Pharmacol*., 70(6), 949-958 (2005).

102. Chaudhary PM, Mechetner EB, Roninson IB. Expression and activity of the multidrug resistance P-glycoprotein in human peripheral blood lymphocytes. *Blood*, 80(11), 2735-2739 (1992).

103. Cattaneo D, Ruggenenti P, Baldelli S *et al*. ABCB1 genotypes predict cyclosporine-related adverse events and kidney allograft outcome. *Journal of the American Society of Nephrology : JASN*, 20(6), 1404-1415 (2009).

104. Crettol S, Venetz JP, Fontana M *et al*. Influence of ABCB1 genetic polymorphisms on cyclosporine intracellular concentration in transplant recipients. *Pharmacogenetics and genomics*, 18(4), 307-315 (2008).

105. Ansermot N, Rebsamen M, Chabert J *et al*. Influence of ABCB1 gene polymorphisms and P-glycoprotein activity on cyclosporine pharmacokinetics in peripheral blood mononuclear cells in healthy volunteers. *Drug metabolism letters*, 2(2), 76-82 (2008).

106. Capron A, Mourad M, De Meyer M *et al*. CYP3A5 and ABCB1 polymorphisms influence tacrolimus concentrations in peripheral blood

mononuclear cells after renal transplantation. *Pharmacogenomics*, 11(5), 703-714 (2010).

107. Chen N, Weiss D, Reyes J *et al*. No clinically significant drug interactions between lenalidomide and Pglycoprotein substrates and inhibitors: results from controlled phase I studies in healthy volunteers. *Cancer chemotherapy and pharmacology*, 73(5), 1031-1039 (2014).

108. Anglicheau D, Pallet N, Rabant M *et al*. Role of P-glycoprotein in cyclosporine cytotoxicity in the cyclosporine-sirolimus interaction. *Kidney international*, 70(6), 1019-1025 (2006).

109. Laplante A, Demeule M, Murphy GF, Beliveau R. Interaction of immunosuppressive agents rapamycin and its analogue SDZ-RAD with endothelial P-gp. *Transplantation proceedings*, 34(8), 3393-3395 (2002).

110. Grude P, Boleslawski E, Conti F, Chouzenoux S, Calmus Y. MDR1 gene expression in peripheral blood mononuclear cells after liver transplantation. *Transplantation*, 73(11), 1824-1828 (2002).

111. Goto M, Masuda S, Kiuchi T *et al*. Relation between mRNA expression level of multidrug resistance 1/ABCB1 in blood cells and required level of tacrolimus in pediatric living-donor liver transplantation. *The Journal of pharmacology and experimental therapeutics*, 325(2), 610-616 (2008).

112. Robertsen I, Falck P, Andreassen AK *et al*. Endomyocardial, intralymphocyte, and whole blood concentrations of ciclosporin A in heart transplant recipients. *Transplantation research*, 2(1), 5 (2013).

113. Lemaitre F, Antignac M, Fernandez C. Monitoring of tacrolimus concentrations in peripheral blood mononuclear cells: Application to cardiac transplant recipients. *Clinical biochemistry*, (2013).

114. Ansermot N, Fathi M, Veuthey JL, Desmeules J, Hochstrasser D, Rudaz S. Quantification of cyclosporine A in peripheral blood mononuclear cells by liquid chromatography-electrospray mass spectrometry using a column-switching approach. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 857(1), 92-99 (2007).

115. Falck P, Guldseth H, Asberg A, Midtvedt K, Reubsaet JL. Determination of ciclosporin A and its six main metabolites in isolated T-lymphocytes and whole blood using liquid chromatography-tandem mass spectrometry. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 852(1-2), 345-352 (2007).

116. Brozmanova H, Perinova I, Halvova P, Grundmann M. Liquid chromatography-tandem mass spectrometry method for simultaneous determination of cyclosporine A and its three metabolites AM1, AM9 and AM4N in whole blood and isolated lymphocytes in renal transplant patients. *Journal of separation science*, 33(15), 2287-2293 (2010).

117. Capron A, Lerut J, Latinne D, Rahier J, Haufroid V, Wallemacq P. Correlation of tacrolimus levels in peripheral blood mononuclear cells with histological staging of rejection after liver transplantation: preliminary results of a prospective study. *Transplant international : official journal of the European Society for Organ Transplantation*, 25(1), 41-47 (2012).

118. Capron A, Musuamba F, Latinne D *et al*. Validation of a liquid chromatography-mass spectrometric assay for tacrolimus in peripheral blood mononuclear cells. *Therapeutic drug monitoring*, 31(2), 178-186 (2009).

119. Gustafsson F, Barth D, Delgado DH, Nsouli M, Sheedy J, Ross HJ. The impact of everolimus versus mycophenolate on blood and lymphocyte cyclosporine exposure in heart-transplant recipients. *European journal of clinical pharmacology*, 65(7), 659-665 (2009).

120. Falck P, Asberg A, Guldseth H *et al*. Declining intracellular T-lymphocyte concentration of cyclosporine a precedes acute rejection in kidney transplant recipients. *Transplantation*, 85(2), 179-184 (2008).

121. Lepage JM, Lelong-Boulouard V, Lecouf A, Debruyne D, Hurault de Ligny B, Coquerel A. Cyclosporine monitoring in peripheral blood mononuclear cells: feasibility and interest. A prospective study on 20 renal transplant recipients. *Transplantation proceedings*, 39(10), 3109-3110 (2007).

122. Barbari A, Stephan A, Masri M *et al*. Cyclosporine lymphocyte level and lymphocyte count: new guidelines for tailoring immunosuppressive therapy. *Transplantation proceedings*, 35(7), 2742-2744 (2003).

123. Barbari A, Masri MA, Stephan A *et al*. Cyclosporine lymphocyte versus whole blood pharmacokinetic monitoring: correlation with histological findings. *Transplantation proceedings*, 33(5), 2782-2785 (2001).

124. Masri MA, Barbari A, Stephan A, Rizk S, Kilany H, Kamel G. Measurement of lymphocyte cyclosporine levels in transplant patients. *Transplantation proceedings*, 30(7), 3561-3562 (1998).

125. Masri M, Rizk S, Barbari A, Stephan A, Kamel G, Rost M. An assay for the determination of sirolimus levels in the lymphocyte of transplant patients. *Transplantation proceedings*, 39(4), 1204-1206 (2007).

126. Roullet-Renoleau F, Lemaitre F, Antignac M, Zahr N, Farinotti R, Fernandez C. Everolimus quantification in peripheral blood mononuclear cells using ultra high performance liquid chromatography tandem mass spectrometry. *Journal of pharmaceutical and biomedical analysis*, 66, 278-281 (2012).

127. Starkel P, Sempoux C, Van Den Berge V *et al*. CYP 3A proteins are expressed in human neutrophils and lymphocytes but are not induced by rifampicin. *Life Sci*., 64(8), 643-653 (1999).

128. Dey A, Yadav S, Dhawan A, Seth PK, Parmar D. Evidence for cytochrome P450 3A expression and catalytic activity in rat blood lymphocytes. *Life sciences*, 79(18), 1729-1735 (2006).

129. Hesselink DA, van Schaik RH, van der Heiden IP *et al*. Genetic polymorphisms of the CYP3A4, CYP3A5, and MDR-1 genes and pharmacokinetics of the calcineurin inhibitors cyclosporine and tacrolimus. *Clinical pharmacology and therapeutics*, 74(3), 245-254 (2003).

130. Kamdem LK, Streit F, Zanger UM *et al*. Contribution of CYP3A5 to the in vitro hepatic clearance of tacrolimus. *Clinical chemistry*, 51(8), 1374-1381 (2005).

131. Miura M, Niioka T, Kagaya H *et al*. Pharmacogenetic determinants for interindividual difference of tacrolimus pharmacokinetics 1 year after renal transplantation. *J*. *Clin*. *Pharm*. *Ther*., 36(2), 208-216 (2011).

132. Uesugi M, Masuda S, Katsura T, Oike F, Takada Y, Inui K. Effect of intestinal CYP3A5 on postoperative tacrolimus trough levels in living-donor liver transplant recipients. *Pharmacogenetics and genomics*, 16(2), 119-127 (2006).

133. Lensmeyer GL, Wiebe DA, Carlson IH, Subramanian R. Concentrations of cyclosporin A and its metabolites in human tissues postmortem. *Journal of analytical toxicology*, 15(3), 110-115 (1991).

134. Sandborn WJ, Lawson GM, Cody TJ *et al*. Early cellular rejection after orthotopic liver transplantation correlates with low concentrations of FK506 in hepatic tissue. *Hepatology (Baltimore, Md.)*, 21(1), 70-76 (1995).

135. Sandborn WJ, Lawson GM, Krom RA, Wiesner RH. Hepatic allograft cyclosporine concentration is independent of the route of cyclosporine administration and correlates with the occurrence of early cellular rejection. *Hepatology (Baltimore, Md.)*, 15(6), 1086-1091 (1992).

136. Capron A, Lerut J, Verbaandert C *et al*. Validation of a liquid chromatography-mass spectrometric assay for tacrolimus in liver biopsies after hepatic

transplantation: correlation with histopathologic staging of rejection. *Therapeutic drug monitoring*, 29(3), 340-348 (2007).

137. Noll BD, Coller JK, Somogyi AA *et al*. Measurement of cyclosporine A in rat tissues and human kidney transplant biopsies--a method suitable for small (<1 mg) samples. *Therapeutic drug monitoring*, 33(6), 688-693 (2011).

138. Noll BD, Coller JK, Somogyi AA *et al*. Validation of an LC-MS/MS Method to Measure Tacrolimus in Rat Kidney and Liver Tissue and Its Application to Human Kidney Biopsies. *Therapeutic drug monitoring*, (2013).

139. Ting LS, Partovi N, Levy RD, Riggs KW, Ensom MH. Pharmacokinetics of mycophenolic acid and its phenolic-glucuronide and ACYl glucuronide metabolites in stable thoracic transplant recipients. *Therapeutic drug monitoring*, 30(3), 282-291 (2008).

140. Elens L, Capron A, Kerckhove VV *et al*. 1199G>A and 2677G>T/A polymorphisms of ABCB1 independently affect tacrolimus concentration in hepatic tissue after liver transplantation. *Pharmacogenetics and genomics*, 17(10), 873-883 (2007).

141. Smans L, Lentjes E, Hermus A, Zelissen P. Salivary cortisol day curves in assessing glucocorticoid replacement therapy in Addison's disease. *Hormones (Athens, Greece)*, 12(1), 93-100 (2013).

142. Kovarik JM, Sabia HD, Figueiredo J *et al*. Influence of hepatic impairment on everolimus pharmacokinetics: implications for dose adjustment. *Clinical pharmacology and therapeutics*, 70(5), 425-430 (2001).

143. Peveling-Oberhag J, Zeuzem S, Yong WP *et al*. Effects of hepatic impairment on the pharmacokinetics of everolimus: a single-dose, open-label, parallel-group study. *Clinical therapeutics*, 35(3), 215-225 (2013).

144. Reece PA, Disney AP, Stafford I, Shastry JC. Prednisolone protein binding in renal transplant patients. *British journal of clinical pharmacology*, 20(2), 159-162 (1985).

145. MacDonald A, Scarola J, Burke JT, Zimmerman JJ. Clinical pharmacokinetics and therapeutic drug monitoring of sirolimus. *Clinical therapeutics*, 22 Suppl B, B101-121 (2000

Chapter 2 : MANUSCRIPT II

To be submitted to Journal of Chromatography B

Development and Validation of Sensitive and Selective LC-MS/MS Method for Quantification of Tacrolimus in Oral Fluid Samples from Kidney Transplant Recipients

AUTHORS

[1]Mwlod Ghareeb, [1]Fatemeh Akhlaghi.

DEPARTMENT AND INSTITUTION

[1]Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, RI

Address correspondence to: Fatemeh Akhlaghi, PharmD, PhD, Clinical Pharmacokinetics Research Laboratory, Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, 495A College of Pharmacy, 7 Greenhouse Road, Kingston, RI 02881, USA. Phone: (401) 874 9205. Fax: (401) 874 5787. Email: fatemeh@uri.edu

KEYWORDS

Renal transplantation, tacrolimus, saliva, oral fluid, therapeutic drug monitoring, immunosuppressant, LC-MS/MS.

ABBRIVATIONS

ACN: Acetonitrile, ASC: Ascomycin, CV: Coefficient of variation, DBS: Dried blood spot, ESI: Electrospray ionization, FDA: Food and Drug Administration, IS: Internal standard, ISR: Incurred Sample Reanalysis, LC-MS/MS: Liquid chromatography tandem mass spectrometry, LLOQ: Lower limit of quantification, ME: Matrix effect, MeOH: Methanol, MRM: Multiple reaction monitoring, MS: Mass spectrometry, MW: molecular weight, OF: Oral fluid, QCs: Quality controls, RTR: Renal transplant recipients, S/N: Signal to noise ratio, TAC: Tacrolimus, TDM: Therapeutic Drug Monitoring.

CORRESPONDING AUTHOR

Address correspondence to: Fatemeh Akhlaghi, PharmD, PhD, Clinical Pharmacokinetics Research Laboratory, Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, 495A College of Pharmacy, 7 Greenhouse Road, Kingston, RI 02881, USA. Phone: (401) 874 9205. Fax: (401) 874 5787. Email: fatemeh@uri.edu

DISCLOSURE

No conflict of interest is declared.

ABSTRACT

Tacrolimus (TAC) is a commonly used immunosuppressive agent in solid organs transplant recipients. Due to its high inter-subject and intra-subject variability and the consequent risk of toxicity and/or allograft rejection, therapeutic drug monitoring (TDM) is required. Venipuncture blood sampling is recommended for tacrolimus TDM. Using peripheral blood samples for quantifying TAC concentration has limitations of being invasive and provides poor correlation with TAC concentration at the site of action, in lymphocytes and tissues. Tacrolimus concentration that is present in oral fluid (OF) is considered representative of the free fraction that is responsible for the desired clinical outcomes and toxicity. Therefore, measuring salivary TAC may provide a suitable alternative to using whole blood. In this study, a validated, rapid, sensitive and selective liquid chromatography tandem mass spectrometry (LC-MS/MS) is presented. Chromatography separation was achieved using Acquity UPLC BEH C18 column and gradient elution using 2mM ammonium acetate/0.1 formic acid in water (mobile phase A) and in methanol (mobile phase B). Short sample analysis cycle with 2.2 min run was achieved. Simple sample preparation and extraction procedure with two folds of ACN as precipitating solvent provided sufficient sample cleanness and negligible matrix effect. Tacrolimus was stable in OF for up to one month at -80 °C and up to 48hr in auto-sampler at 20 °C. The method showed high reproducibility as confirmed by incurred sample reanalysis (ISR) test.

Introduction

Tacrolimus (TAC) is a widely described immunosuppressive agent for solid organ transplant recipients. It has a narrow therapeutic index, significant intra- [146] and inter-subject pharmacokinetic variability [3]. Therefore, routine TDM is required for optimized outcomes [147]. Currently, peripheral venous blood is recommended for estimation of TAC exposure [148]. However, TAC blood level showed poor correlation with, in situ level, in lymphocytes [146] and tissues [146] as well as free fraction [146]. As a result, whole blood sampling fails to provide a reliable prediction of allograft rejection and toxicity [146]. Moreover, the invasive nature of venipuncture sampling makes this approach less appealing as compared to less invasive sampling methods [15][146].

Intra-lymphocytes and intra-tissue TAC concentration showed to be good indicators of therapeutic efficacy and predictor for allograft rejection in liver [146] and kidney [146] transplant recipients. However, using these techniques in clinical practice are hampered by relatively large amount of blood needed for intracellular TAC measurement (7-8 mL); laborious lymphocytes isolation and TAC extraction procedures; and the need for biopsy for intra-tissue.

Dried blood spot (DBS) sampling was proposed as a possible less invasive alternative to venipuncture [146]. Dried blood spot provides a way of obtaining samples less invasively and makes patient self-sampling more feasible [146]. Tacrolimus concentration obtained using DBS showed excellent association with TAC venous level [146]. However, since whole blood is used in preparing DBS, it still has the main

disadvantage of the venous blood of being a poor predictor for intra-lymphocytes and intra-tissue concentration.

For TAC, about 1% of the total amount in blood present in free form, which is responsible for clinical outcome and toxicity [3]. The free fraction significantly affected by changes in plasma lipoprotein concentration after transplantation [146], leading to the incidence of episodes of rejection and/or toxicity [146]. Therefore, probably it is sensible to monitor free TAC concentration instead of total concentration.

Accumulated knowledge about how drugs partition into saliva promoted OF as possible media for TDM [146]. Due to their large size, protein-bound drugs are unable to cross biological membranes, and the only free fraction of a drug can enter OF [146]. Therefore, drugs concentration in OF represents free drug fraction [35]. Accordingly, OF may enable measuring free drug fraction directly in samples obtained easily and non-invasively with minimum sample preparation steps [146]. In this paper, a validated, rapid, sensitive and selective method for quantifying TAC in OF is presented. The study also investigated optimum sample treatment conditions using simple extraction methods.

Chemicals and reagents

Tacrolimus ($C_{44}H_{69}NO_{12}$, MW = 804.02, 1.0 mg/mL solution in acetonitrile) and the internal standard (IS), ascomycin (ASC, $C_{43}H_{69}NO_{12}$, Mw = 792.01, 1.0 mg/mL solution in acetonitrile) were purchased from Cerilliant Corporation (Round Rock, Texas, USA). Optima LC/MS grade of acetonitrile (ACN), ammonium acetate, formic

acid, and methanol (MeOH) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was obtained using Milli-Q Synthesis system fitted with Q-Gard 2 Purification Pack (Millipore, Bedford, MA, USA). AquaSil Siliconizing Fluid was purchased from Thermo Fisher Scientific Inc (Franklin, MA, USA). Drug-free human OF from six donors was obtained from Bioreclamation Inc. (Westbury, NY, USA).

Apparatus

Samples were sonicated using Branson® Sonicator (Danbury, CT, USA) to produce a homogeneous mixture. The supernatant was obtained using Eppendorf 5810 centrifuge from Micro and Nanotechnology (Urbana, IL, USA). Samples were analyzed using Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS). The LC-MS/MS system consisted of Acquity UPLC from Waters Corp (Milford, MA, USA) connected to Xevo TQ MS mass spectrometry from Waters Corp. MassLynx™ software (V 4.1) was used to control the system and data acquisition, and data processed using TargetLynx™ tool. The UPLC system had a binary pump and equipped with built-in column heater. Twenty micro-litters sample loop was used to deliver 10 µL of the samples in partial loop mode. For salivary blood contamination assay, SpectraMax M5e Microplate Reader (Sunnyvale, CA, USA) was used.

Chromatographic conditions

An Acquity UPLC BEH C18 (2.1 mm x 50 mm) column with 1.7µm-particle size and 130Å pores size used (Waters Corp) for chromatographic separation. An Acquity

UPLC BEH C18, (2.1 mm x 5 mm) pre-column with 1.7µm particle size and 130Å porosity (Waters Corp) was connected immediately to the inlet of the analytical column. The temperature of the column was kept at 60 °C, and the auto-sampler temperature was maintained at 20 °C. Gradient elution was employed with a mobile phase consisted of water containing 2 mM ammonium acetate/0.1% (v/v) formic acid (Solvent A); and MeOH containing 2 mM ammonium acetate/0.1% (v/v) formic acid (Solvent B). The mobile phase was delivered at 0.4mL/ min flow rate. The run cycle started at 50% solvent (B) and increased gradually to 98% over 0.5 min and maintained at this level till 1.8 min. To re-equilibrate the column for next run, solvent (B) decreased within 0.1 min to 50% and kept till the end of the run at 2.2 min. Diversion valve was set to deliver the first 0.70 min and from 1.20 min till the end of each run to waste. The elution time of ASC and TAC was 1.0 min.

Mass spectrometry condition

Mass spectrometry detection and quantification of TAC and ASC performed in positive electrospray ionization (ESI) and multiple reaction monitoring (MRM) modes. Intellistart tool was used to obtain initial mass spectrometry parameters in low mass resolution analysis mode followed by manual tuning to achieve highest possible sensitivity. Final mass spectrometry parameters were as following: collision energy (CE) = 22 and 20 for ASC and TAC respectively, cone voltage (CV) = 28, capillary voltage (kV = 1.50), source temperature (°C) = 150, cone gas flow (L/hr) = 25, desolvation gas flow (L/hr) = 1000, and collision gas flow (mL/min) = 0.15. Ammonium adducts [M+NH4]+ were selected as precursors for MRM with transitions

(m/z, Q1 > Q3) of (m/z, 809.30 > 756.30) and (m/z, 821.30 > 768.35) for ASC and TAC, respectively.

Standards, quality controls, and internal standard solutions preparation

Sub-stock and working stock solutions of ASC and TAC were prepared from the original solutions (1mg/mL) using ACN and MeOH, respectively, and stored at – 20 °C. Standards and quality controls (QCs) were prepared by spiking the OF with serially diluted working stock solutions (< 5% of total OF volume) to achieve desired concentrations. A final concentration of 600 ng/L ASC in ACN was used as the precipitating solvent.

Patients Samples

Studies protocols approved by Institutional Review Board at Rhode Island Hospital (Providence, RI). After giving the formal consent, kidney transplant recipients attending kidney transplant clinics were recruited. All patients were on a triple immunosuppressive regimen including tacrolimus, prednisone, and mycophenolic or azathioprine). After the physical examination by the physician, signed inform consent was obtained from each patient. In two studies, patients were asked to give venous blood samples (approximately 4 mL collected ethylenediaminetetraacetic acid (EDTA) and matching OF samples. In the first study, 85 samples were collected sporadically at certain time points, including, pre-dose (time 0 = C_0) and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10 and 12 hrs post-dose from 10 patients. In the second study, samples collected at 0 hr (50 samples) and 2 hrs from (46 samples) from 61 patients. The OF

samples were collected by passive drool into siliconized plastic cups. All blood and OF samples kept on dry ice till transferred to the Department of Biomedical and Pharmaceutical Sciences (BPS) at the University of Rhode Island and stored at – 80 °C until analyzed.

Sample extraction

Calibration standards, quality controls (QCs), blank, and patients' OF samples were allowed to thaw at room temperature. After vortexing for 5 seconds, samples were sonicated for 5-10 seconds (depending on samples volume) to breakdown salivary components and produce a homogenous mixture. Fifty micro-liters of the samples were transferred into a 1.5 mL polypropylene tube, and 100 µL of precipitating solvent were added (IS final concentration was 200 ng/L). After vortexing for 10 seconds, samples centrifuged at 10,000 xg for 5 min at 20 °C. The supernatant were then transferred into an auto-sampler vial and 10 µL was injected.

Statistical analysis of the data

Statistical analysis was performed using the SPSS software (version 19.0, SPSS Inc., Chicago, IL, USA) and GraphPad Prism (version 4.0, GraphPad Software, Inc., La Jolla, CA, USA). Normal distribution of the data was checked graphically and confirmed with the Shapiro-Wilk test.

Assay validation

Standards and QCs

The method was validated in accordance with the current version of FDA guidance for industry on bioanalytical method validation [149]. Tacrolimus to internal standard peak ratio against tacrolimus nominal concentration was used to construct the calibration curve and fitted using (1/x) weighting method. Calibration curve concentrations were 10, 20, 50, 100, 250, 750, 1440, and 1600 ng/L. Quality control concentrations were set at 30, 200, and 1200 ng/L. To determine accuracy and precision of the assay, three different batches of OF were spiked with the working stocks solution to achieve standards and QCs (6 replicate) concentrations and extracted as described in sample extraction section.

Sensitivity and selectivity

Lower limit of quantification (LLOQ) was set at the concentration with a signal to noise ratio (S/N) of at least 10, accuracy between 80-120%, and Coefficient of Variation (CV) less than 20%. Acceptance criteria for QCs were accuracy between 85-115% and CV less than 15%. Selectivity was assessed by inspecting the presence of noise or peaks in chromatograms that represented blank OF samples (from 6 donors) as compared with LLOQ sample chromatogram.

Stability

Stability studies were performed by measuring TAC concentrations in QC1 and QC3, in three replicate. Freeze and thaw (after three freeze and thaw cycles), bench-top, auto-sampler (by re-injecting one of validation batch after it was left in the auto-

sampler for 24 hr and 48 hr), and short-term stability up to one month were investigated.

Matrix effect and Recovery

The presence and possible matrix effect (ME) in OF studied in two different ways. In the first approach, chromatograms obtained from post-column infusion test were inspected visually. This test involved continuous infusion of 98% methanol (which represents the composition of mobile phase at elution time of ASC and TAC) containing 1 ng/mL of ASC and TAC at 20 µL/min flow rate after the column through a Tee connection. After establishing the baseline, a 10 µL of blank extracted OF sample was injected using the pre-established LC method. The resulting chromatogram was checked for symptom of ion suppression and/or enhancement in comparison to blank injection of neat solution (1:2, water:ACN). In the second approach, possible interference of OF components, namely the phospholipids, was studied. As such, MRM transitions of abundant phospholipids were added to MS method to enable us to visually locate their elution region.

The effect of increasing ratio of precipitating solvents on the ME was also studied to select the ratio that offers best sample cleanness. Two different sets of QC1 and QC3 samples were prepared, in triplicate, either by (i) QCs samples (set 1) prepared by adding ACN to OF samples spiked with TAC as prescribed in sample extraction section (pre-extraction spiked samples); (ii) QCs samples (set 2) prepared using a mixture of de-ionized water: ACN (neat solution). In each set, different ratios of ACN were added (1:1, 1:2 and 1:3). In total, 18 samples were analyzed, 9 samples in each

set. The absolute ME was measured by calculating the percentage of the ratio of mean peaks area of pre-extracted samples to samples prepared in de-ionized water/ACN mixture.

Recovery was assessed by analyzing a third set of QCs samples (set 3) prepared by extracting blank OF first with 1:1, 1:2 and 1:3 ACN, followed by adding TAC working standard solutions to achieve required concentrations (post-extraction spiked samples). The recovery was determined by calculating the percentage of the ratio of mean peaks area of pre-extraction samples (set 1) to post-extracted spiked samples (set 3).

Results and discussion

Recommended TAC C_0 therapeutic blood concentration in kidney transplant recipients is between 15-20 µg/L immediately after transplantation [3]. TAC dose is tapered gradually, and the maintenance C_0 can be as low as 5-7 µg/L after first year post-transplantation [3]. Since only 1% of TAC amount found in the unbound form that is capable of reaching the OF, the expected OF concentration would range between 0.050-200 ng/L. Therefore, highly optimized mass spectrometry and chromatographic conditions were sought to develop a method with adequate selectivity and sensitivity. To achieve the highest selectivity feasible, different columns were tested. Acquity UPLC BEH C18 seemed to be a good choice as it gave sharp and symmetric peaks. Given the above-mentioned UPLC and mass spectrometry conditions, it was possible to set LLOQ at 10 ng/L with signal/noise ratio of more than 10 (Figure 1A). No carryover was detected when a double blank OF sample was injected following

highest calibration concentration (Figure 1B). The calibration curve was constructed by plotting nominal standards concentration against peak area ratios of the analyte to IS and fitted with 1/x weighted least squares linear regression. The method demonstrated adequate accuracy and precision with QCs accuracy between 94.5-103.6%, and CV within 4– 9.8 (Table 1). The correlation coefficients (r^2) calculated from validation batches (n=3) were between 0.998-0999.

Stability studies, namely, freeze and thaw, bench top, auto-sampler, and short-term storage at –80 °C for up to four weeks were conducted (Table 2). No stability problems were noticed, and TAC was stable in extracted matrix for up to 48 hrs.

Possible interference from endogenous substances in OF was investigated. Chromatograms obtained from acquiring a pooled blank OF from six donors (Figure 1B) and blank neat solution (66% ACN) (Figure 1C). No signs of interference were noticed.

Using methanol instead of ACN as organic solvent helped improving the sensitivity, In addition, LC/MS grade methanol showed to boost the sensitivity by about 20%. Positive mode ionization and monitoring ammonium adduct [M + NH4]+ at (m/z, 821.30 > 768.35) provided better signal compared to [M]+ and [M + Na]+.

Matrix effect and recovery

Co-eluting of drug with endogenous substance in OF may lead to either ion suppression or enhancement, which collectively named as ME [146]. The presence of ME could compromise the reproducibility and may lead to data bias [150]. Different cleaning procedures were used in methods aimed to measure the immunosuppressive agents OF samples. These techniques included solid phase extraction [146], analytes

concentrating by drying and reconstituting [146] and simple protein precipitation using organic solvents [146]. Type and percentage of precipitating solution could have an effect on sensitivity and selectivity through its effect on the yield of analytes and cleanness of extracted sample. Acetonitrile has been reported to provide satisfactory protein precipitation in oral fluid samples [146]. Recovery of some drugs and ME achieved using MeOH and ACN as precipitating solvent in plasma are comparable; however, MeOH tends to retain about 40% more phospholipids [146]. Therefore, ACN was chosen as extracting solvent. Belostotsky, et al. [146] used 1:3, ACN:OF to measure TAC salivary concentration. In previous studies to quantify immunosuppressants simple protein precipitation in OF using, 1:2 and 1:3, OF: ACN was used for mycophenolic acid (MPA) and TAC, respectively. To the authors' knowledge, no study was published to date that has investigated the optimal proportion of extracting solvent (ACN) that gives maximum recovery and sample cleaning up. To examine the effect of using different proportions of ACN on recovery and absolute ME, OF samples were extracted with an equal, double and triple amount of ACN (Table 3). As it can be seen from the table, there was slightly less variability in the areas count in samples extracted with double volume of ACN compared to other two categories. Standard deviations were ± 7- 577, ± 4- 138 and ± 11.5- 141.6 for OF extracted with the equal, double and 3 times volume ACN, respectively. The recovery ranged between (101.6 - 112.7), (100.0 - 113.8), and (113.8 - 124.3); and ME was within (79.8 – 93.2), (95.6-116.0) and (100.9 – 131.3) for 1:1, 1:2 and 1:3 OF: ACN, respectively. Based on these values, it is obvious that samples extracted with three-folds ACN gave over estimated recoveries while other two groups showed comparable

recovery ranges. For ME, the first group showed to have significant ion suppression of about 20% in QC1 samples. Based at the variably of the acquired data, adequate sample cleaning, recovery, and minimum sample dilution, two-folds of ACN was chosen for protein precipitation.

Matrix effect was also explored visually using post-column infusion technique [146]. The composite chromatograms in figures 2A and 2B was obtained by overlying chromatograms acquired from injecting neat solution (66% ACN), blank OF with continues infusion of a mixture of ASC and TAC (1 µg/L) and a chromatogram of QC2 injection. The only areas of chromatograms that show ion suppression are between 0.2-0.5 min, which is far enough from ASC and TAC elution area.

Finally, potential co-elution of phospholipids was examined by adding MRM of transitions of most common ones to the mass spectrometry method [146]. Phospholipids transitions included were (m/z, 496 > 184, 520 > 184, 522> 184, 524 > 184, 758 > 184, 782 > 184). In early stages of method development, ASC and TAC peaks co-eluted with low molecular weight phospholipids (m/z 496 and 524). By manipulating mobile phase gradient, a full separation between analytes of interest and the phospholipids was achieved (Figure 3). The other two phospholipids that have m/z > 700 were less problematic and eluted way after analytes of interest.

In total 181 samples collected from 71 kidneys transplant patients analyzed only one sample had concentration lower than LLOQ with calculated concentration around 8.5 ng/L, collected 2 hr after dose, even with the corresponding blood concentration was within the normal range (11.8 µg/L). The concentration of TAC ranged from 11.7-

2864.4 ng/L and 1.7- 46.06 µg/L for OF and whole blood, respectively. The clinical finding of this study will be presented in a separate manuscript.

Incurred sample re-analysis

The incurred samples reanalysis test was performed by re-analyzing about 10% of the samples (19 samples) [146]. Whenever many samples were available per patient, two samples were selected to represent absorption and elimination phases. The difference between the paired measurements were normally distributed, therefore, the use of Bland–Altman method was justified [151]. Repeatability was tested visually (Figure 3) and statistically. Good agreement between the two repeated measurements can be observed in Figure 3, which plots the percent differences between paired repeated measurements against their mean. All points lie between or near the 95% confident interval lines. The 95% limit of the agreement was from – 19.16 to 31.98. The bias (mean the difference between two occasions) was 6.40.

Conclusion

In this paper, development and validation of a very sensitive, selective and robust method is presented. Simple sample preparation and extraction protocol was developed and used to provide minimum sample dilution and appropriate samples cleanliness, excellent recovery and minimum sample components interference. In addition to lowest reported LLOQ of TAC, this work is the first to study the effect of different proportions of precipitating solvent (ACN) on the ME and recovery. In addition, this is the first report that investigated and described phospholipids

chromatographic elution behavior and the possible interference of phospholipids with the analyte in the OF.

Table 2-1: Summary of QC samples from three individual runs (mean ± % CV, each QC had 6 replicates in each validation run, Total=18)

	LLOQ	**QC1**	**QC2**	**QC3**
Quality control samples (pg/mL)	10	30	200	1200
% Accuracy	103.6	103.0	94.5	99.4
% CV	9.8	6.1	5.9	4.0

Accuracy = (mean concentration/nominal concentration) × 100, % CV = (standard deviation/mean) × 100.

Table 2-2: Results of stability studies (mean ± % CV, N= 3).

QCs (pg/mL)	Bench top	Freeze thaw	& Auto-sampler		Short-term	
			24 hrs	48 hrs	1 weeks	4 weeks
QC1 (30) Accuracy (CV)	111.1 (9.4)	108.2 (5.8)	105.3 (9.6)	102.5 (4.3)	98.4 (11.9)	100.8 (6.7)
QC3 (1200) Accuracy (CV)	98.8 (2.4)	111.9 (2.0)	102.2 (2.7)	105.6 (3.6)	98.9 (3.4)	102.9 (1.9)

Table 2-3: Effect of different ratios of oral fluid sample: extraction solvent (ACN) on recovery and absolute matrix effect, expressed as mean peak area ± std (n =3)

	QC1 (30 pg/mL)			QC2 (200 pg/mL)			QC3 (1200 pg/mL)		
Matrix	**1:1**	**1:2**	**1:3**	**1:1**	**1:2**	**1:3**	**1:1**	**1:2**	**1:3**
De-ionized water	104.0 (7.5)	±91.6 ± (4.0)	85.0 (14.7)	±1592 ± (44.5)	1738.6 ± (50.3)	1719.0 (141.6)	±4260.0 ± (577.0)	4653.0 ± (109.1)	4409.3± (138.5)
Extracted OF	83.0 ± (7.0)	106.3 ± (8.1)	111.6 ± (13.5)	1485.3 ± (138.5)	1688.6 ± (53.6)	1774.6 ± (47.1)	3916.3 ± (139.1)	4452.3 ± (138,5)	4452.3 ± (63.3)
Post-extraction Spiked OF	81.6 ± (7.7)	106.3 ± (5.6)	96 ± (11.5)	1317.3 ± (36.4)	1483.6 ± (66.5)	1559.0 ± (67)	3759.6 ± (244.7)	4090.3 ± (20.2)	3579.3 ± (59.2)
Recovery (%)	101.6	100.0	116.3	112.7	113.8	113.8	104.1	108.8	124.3

Figure 2-1 Chromatograms of TAC at LLOQ (10pg/mL) (1A, upper) and the internal standard ASC (200pg/mL) (1A,lower). Chromatograms 1B and (1C) represent a pooled blank OF and a blank solvent samples, respectively, injected following highest calibration curve concentration (1600pg/mL) injection.

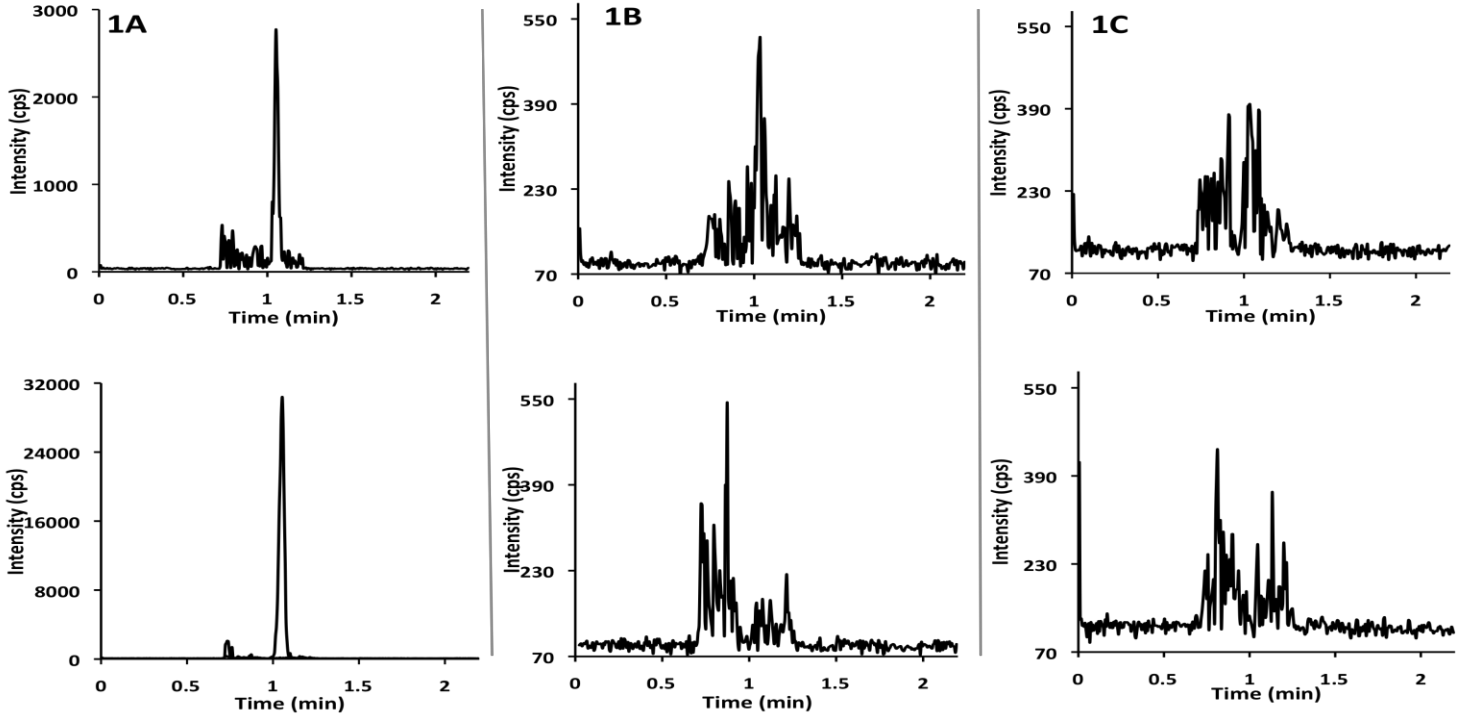


Figure 2-2: Effect of blank OF and blank solvent injections on chromatograms obtained from continues post-column infused mixture of TAC and ASC overlaid on TAC at QC2 concentration (200pg/mL) (2A) and ASC (2B).

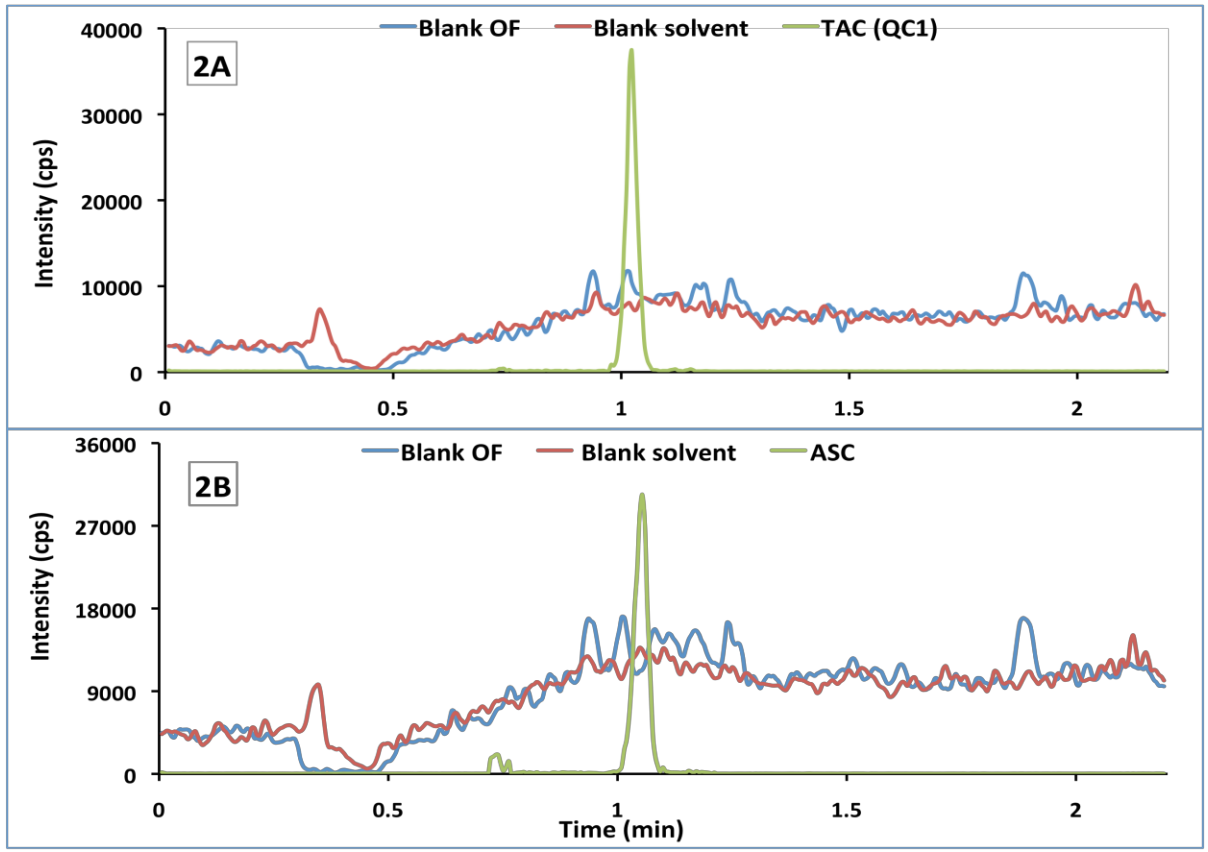


Figure 2-3 A Composite chromatogram shows traces of MRM transitions of 6 major phospholipids, obtained from a chromatogram of extracted blank pooled OF injection overlaid on TAC injection at concentration.

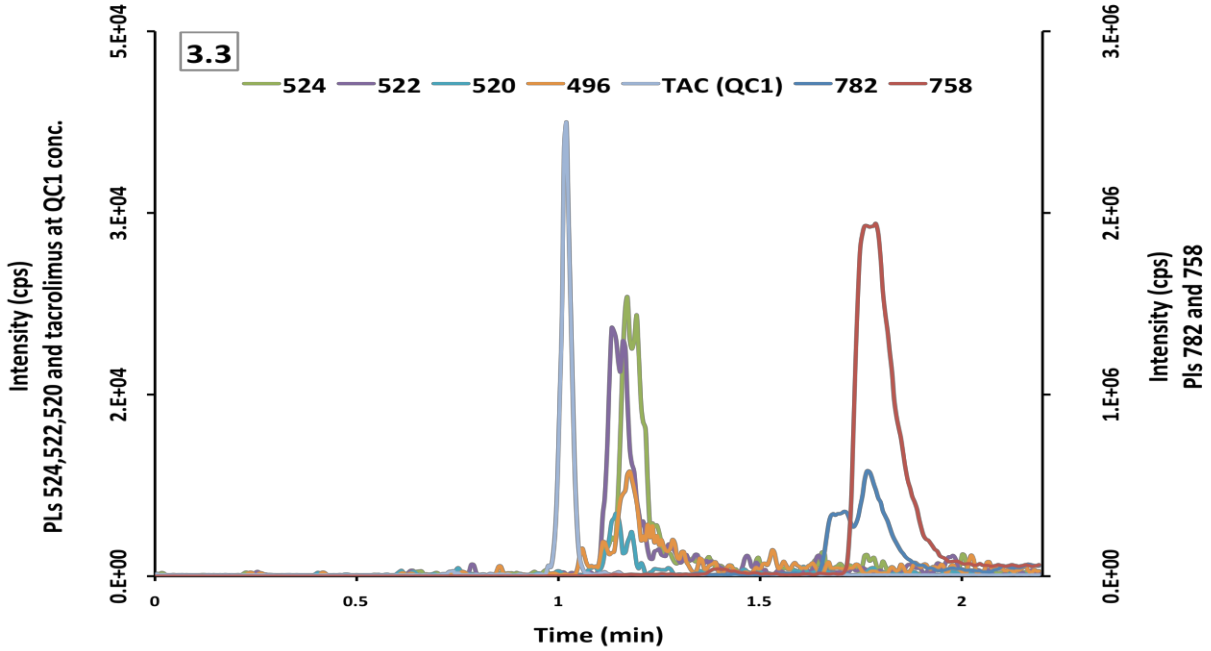
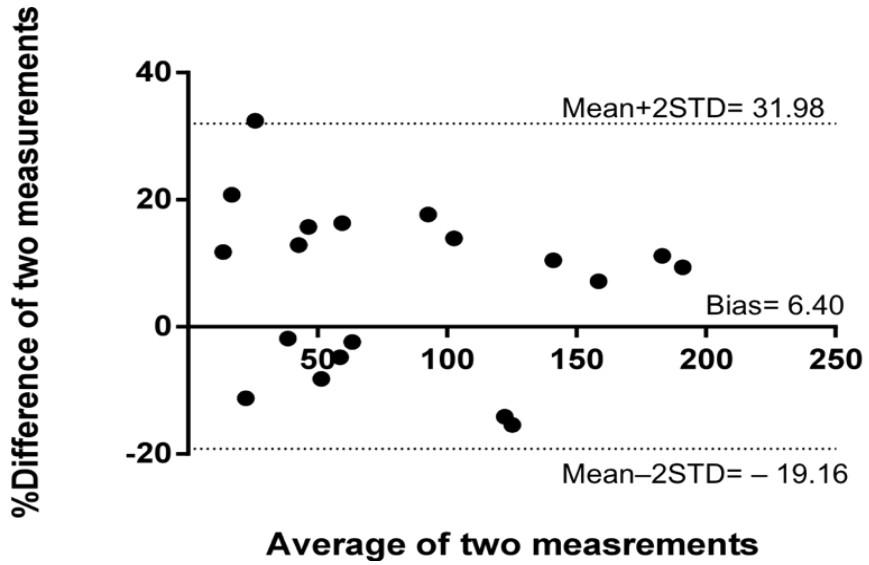


Figure 2-4: Bland-Altman plot of % difference between the repeated measurements plotted against mean differences



References

1. Borra, L.C., et al., *High within-patient variability in the clearance of tacrolimus is a risk factor for poor long-term outcome after kidney transplantation*. Nephrol Dial Transplant, 2010. 25(8): p. 2757-63.

2. Staatz, C.E. and S.E. Tett, *Clinical pharmacokinetics and pharmacodynamics of tacrolimus in solid organ transplantation*. Clin Pharmacokinet, 2004. 43(10): p. 623-53.

3. Scott, L.J., et al., *Tacrolimus: a further update of its use in the management of organ transplantation*. Drugs, 2003. 63(12): p. 1247-97.

4. Taylor, P.J., et al., *The current role of liquid chromatography-tandem mass spectrometry in therapeutic drug monitoring of immunosuppressant and antiretroviral drugs*. Clin Biochem, 2011. 44(1): p. 14-20.

5. Lemaitre, F., M. Antignac, and C. Fernandez, *Monitoring of tacrolimus concentrations in peripheral blood mononuclear cells: Application to cardiac transplant recipients*. Clin Biochem, 2013.

6. Capron, A., et al., *Correlation of tacrolimus levels in peripheral blood mononuclear cells with histological staging of rejection after liver transplantation: preliminary results of a prospective study*. Transpl Int, 2012. 25(1): p. 41-7.

7. Sandborn, W.J., et al., *Early cellular rejection after orthotopic liver transplantation correlates with low concentrations of FK506 in hepatic tissue*. Hepatology, 1995. 21(1): p. 70-6.

8. Zahir, H., et al., *Changes in tacrolimus distribution in blood and plasma protein binding following liver transplantation*. Ther Drug Monit, 2004. 26(5): p. 506-15.

9. Zahir, H., et al., *Factors affecting variability in distribution of tacrolimus in liver transplant recipients*. Br J Clin Pharmacol, 2004. 57(3): p. 298-309.

10. Leichtle, A.B., et al., *Potential of dried blood self-sampling for cyclosporine c(2) monitoring in transplant outpatients*. J Transplant, 2010. 2010: p. 201918.

11. Tennison, M., et al., *Feasibility and acceptance of salivary monitoring of antiepileptic drugs via the US Postal Service*. Ther Drug Monit, 2004. 26(3): p. 295-9.

12. Capron, A., et al., *CYP3A5 and ABCB1 polymorphisms influence tacrolimus concentrations in peripheral blood mononuclear cells after renal transplantation*. Pharmacogenomics, 2010. 11(5): p. 703-14.

13. Capron, A., et al., *Validation of a liquid chromatography-mass spectrometric assay for tacrolimus in peripheral blood mononuclear cells*. Ther Drug Monit, 2009. 31(2): p. 178-86.

14. Noll, B.D., et al., *Validation of an LC-MS/MS Method to Measure Tacrolimus in Rat Kidney and Liver Tissue and Its Application to Human Kidney Biopsies*. Ther Drug Monit, 2013.

15. Elens, L., et al., *1199G>A and 2677G>T/A polymorphisms of ABCB1 independently affect tacrolimus concentration in hepatic tissue after liver transplantation*. Pharmacogenet Genomics, 2007. 17(10): p. 873-83.

16. Capron, A., et al., *Validation of a liquid chromatography-mass spectrometric assay for tacrolimus in liver biopsies after hepatic transplantation: correlation with histopathologic staging of rejection*. Ther Drug Monit, 2007. 29(3): p. 340-8.

17. Hoogtanders, K., et al., *Dried blood spot measurement of tacrolimus is promising for patient monitoring*. Transplantation, 2007. 83(2): p. 237-8.

18. Hoogtanders, K., et al., *Therapeutic drug monitoring of tacrolimus with the dried blood spot method*. J Pharm Biomed Anal, 2007. 44(3): p. 658-64.

19. Cheung, C.Y., et al., *Dried blood spot measurement: application in tacrolimus monitoring using limited sampling strategy and abbreviated AUC estimation*. Transpl Int, 2008. 21(2): p. 140-5.

20. Koop, D.R., et al., *Analysis of tacrolimus and creatinine from a single dried blood spot using liquid chromatography tandem mass spectrometry*. J Chromatogr B Analyt Technol Biomed Life Sci, 2013. 926: p. 54-61.

21. Hinchliffe, E., J.E. Adaway, and B.G. Keevil, *Simultaneous measurement of cyclosporin A and tacrolimus from dried blood spots by ultra high performance liquid chromatography tandem mass spectrometry*. J Chromatogr B Analyt Technol Biomed Life Sci, 2012. 883-884: p. 102-7.

22. den Burger, J.C., et al., *Analysis of cyclosporin A, tacrolimus, sirolimus, and everolimus in dried blood spot samples using liquid chromatography tandem mass spectrometry*. Anal Bioanal Chem, 2012. 404(6-7): p. 1803-11.

23. Webb, N.J., et al., *Fingerprick blood samples can be used to accurately measure tacrolimus levels by tandem mass spectrometry*. Pediatr Transplant, 2005. 9(6): p. 729-33.

24. Yonan, N., et al., *Monitoring of cyclosporine levels in transplant recipients using self-administered fingerprick sampling*. Clin Transplant, 2006. 20(2): p. 221-5.

25. Haeckel, R., *Factors influencing the saliva/plasma ratio of drugs*. Ann N Y Acad Sci, 1993. 694: p. 128-42.

26. Liu, H. and M.R. Delgado, *Therapeutic drug concentration monitoring using saliva samples. Focus on anticonvulsants*. Clin Pharmacokinet, 1999. 36(6): p. 453-70.

27. Haeckel, R. and P. Hanecke, *Application of saliva for drug monitoring. An in vivo model for transmembrane transport*. Eur J Clin Chem Clin Biochem, 1996. 34(3): p. 171-91.

28. Jusko, W.J. and R.L. Milsap, *Pharmacokinetic principles of drug distribution in saliva*. Ann N Y Acad Sci, 1993. 694: p. 36-47.

29. Belostotsky, V., et al., *Measurement of saliva tacrolimus levels in pediatric renal transplant recipients*. Pediatr Nephrol, 2011. 26(1): p. 133-8.

30. Shen, B., et al., *Determination of total, free and saliva mycophenolic acid with a LC-MS/MS method: application to pharmacokinetic study in healthy volunteers and renal transplant patients*. J Pharm Biomed Anal, 2009. 50(3): p. 515-21.

31. *US Department of Health and Human Services. Food and Drug Administration (FDA). Center for Drug Evaluation and Research (CDER). Guidance for Industry.Bioanalytical Method Validation* 2001.

32. Mei, H., et al., *Investigation of matrix effects in bioanalytical high-performance liquid chromatography/tandem mass spectrometric assays: application to drug discovery*. Rapid Commun Mass Spectrom, 2003. 17(1): p. 97-103.

33. Zhang, G. and C.E. Wujcik, *Overcoming ionization effects through chromatography: a case study for the ESI-LC-MS/MS quantitation of a hydrophobic therapeutic agent in human serum using a stable-label internal standard*. J Chromatogr B Analyt Technol Biomed Life Sci, 2009. 877(22): p. 2003-10.

34. Dams, R., et al., *Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: influence of ionization type, sample preparation, and biofluid*. J Am Soc Mass Spectrom, 2003. 14(11): p. 1290-4.

35. Matuszewski, B.K., M.L. Constanzer, and C.M. Chavez-Eng, *Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS*. Anal Chem, 2003. 75(13): p. 3019-30.

36. Mendonza, A.E., R.Y. Gohh, and F. Akhlaghi, *Analysis of mycophenolic acid in saliva using liquid chromatography tandem mass spectrometry*. Ther Drug Monit, 2006. 28(3): p. 402-6.

37. Mendonza, A., R. Gohh, and F. Akhlaghi, *Determination of cyclosporine in saliva using liquid chromatography-tandem mass spectrometry*. Ther Drug Monit, 2004. 26(5): p. 569-75.

38. Wiesen, M.H., et al., *Liquid chromatography-tandem mass spectrometry method for the quantification of mycophenolic acid and its phenolic glucuronide in saliva and plasma using a standardized saliva collection device*. J Chromatogr A, 2012. 1241: p. 52-9.

39. Chambers, E., et al., *Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses*. J Chromatogr B Analyt Technol Biomed Life Sci, 2007. 852(1-2): p. 22-34.

40. Bland, J.M. and D.G. Altman, *Statistical methods for assessing agreement between two methods of clinical measurement*. Lancet, 1986. 1(8476): p. 307-10.

41. van Amsterdam, P., et al., *The European Bioanalysis Forum community's evaluation, interpretation and implementation of the European Medicines Agency guideline on Bioanalytical Method Validation*. Bioanalysis, 2013. 5(6): p. 645-59.

Chapter 3 : MANUSCRIPT III

To be submitted to Clinical Pharmacokinetics

Therapeutic Drug Monitoring of Tacrolimus in Oral Fluids

Mwlod Ghareeb[1], Reginald Y. Gohh [2], Fatemeh Akhlaghi[1]

DEPARTMENT AND INSTITUTION

[1]Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, RI.

[2] Division of Organ Transplantation, Rhode Island Hospital, Warren Alpert Medical School of Brown University, Providence, Rhode Island, USA.

Address correspondence to: Fatemeh Akhlaghi, PharmD, PhD, Clinical Pharmacokinetics Research Laboratory, Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, 495A College of Pharmacy, 7 Greenhouse Road, Kingston, RI 02881, USA. Phone: (401) 874 9205. Fax: (401) 874 5787. Email: fatemeh@uri.edu

DISCLOSURE

No conflict of interest is declared.

ABBREVIATIONS

C_0: Pre dose concentration, C_2: Two hours-post dose concentration, OF: Oral fluid, P-gp: P-glycoprotein transporter, TAC: Tacrolimus, TACs: Tacrolimus salivary concentration, TRNs: Transferrin salivary concentration, TRNs: Transferrin salivary concentration, TDM: Therapeutic drug monitoring

Abstract

Oral fluids, has attracted great attention for therapeutic drug monitoring due to noninvasive nature and the ease of sampling. Considering the only unbound drug can pass through capillaries of salivary gland, salivary drug concentration that is present in can provide an alternative yet convenient specimen for estimating unbound fraction. In this study, the correlation between tacrolimus concentration in oral fluids and blood was investigated. Moreover, factors that may affect such correlation, including sampling time, salivary blood contamination, and food were investigated. In total 256 oral fluid samples with matching blood samples from stable kidney transplant recipients were included in this study. Conclusion: Effect of salivary blood contamination on TACs was minimal when TRN level was ≤ 6.6 mg/dL. Acceptable correlation between oral fluid and blood tacrolimus concentration was observed in fasted samples collected at pre-dose as compared with non-fasted samples collected post dose.

Introduction

To prevent allograft rejection, organ transplant recipients require chronic immunosuppressive therapy [1]. Tacrolimus (TAC) is a widely prescribed immunosuppressive agent for solid organ transplant recipients [1]. It acts by binding to an immunophilin, FK506-binding protein 12 (FKBP12) [2]. The complex then inhibits calcineurin phosphatase and thereby halts T-cell activation [3]. TAC is highly lipophilic and excreted from the body after undergoing extensive metabolism by the CYP450 3A4/5 enzymes [4]. Bioavailability of TAC varies significantly due to genetic polymorphism in CYP3A as well as co-administration of CYP3A enzymes inhibitors or inducers [5-8], thus increasing intra and inter-subject variability in pharmacokinetics [9-12]. In addition, TAC is a substrate for glycoprotein efflux transporter (P-gp), which is also known as multidrug resistance protein 1 (MDR1) encoded by the *ABCB1* gene [13]. Differences in the expression of MDR1 [13] and genotype [14] may contribute to inter-individual variability in tacrolimus pharmacokinetics. Given narrow therapeutic index and high variability, ongoing therapeutic drug monitoring is essential to maintain allograft survival and reduce toxicity [15].

Venipuncture is the recommended medium for TAC therapeutic drug monitoring [1]. However, due the invasive nature of blood sampling, alternative matrices were investigated including dried blood spot and oral fluid (OF) monitoring [16-20]. Oral fluid has attracted great attention as an alternative medium to venipuncture blood [21-23]. The main advantage of OF sampling is noninvasive sample collection, significantly reduced sample collection cost [24, 25] and the possibility of home self-

sampling for patient convenience [24]. In addition, biological barriers are permeable to free drug fraction only considering that protein bound complexes are excluded from passive diffusion because of their large size [26]. Consequently, the portion of a drug that is present in OF represents the unbound fraction [26]. Given that the free fraction is responsible for therapeutic effect and toxicity [27], measuring drug concentration in OF may give better prediction of therapeutic outcomes.

The area under the concentration-time curve (AUC) and maximum concentration (C_{max}) correlate better with clinical outcomes and toxicity that blood sample [28]. Since calculation of AUC requires collection of several samples over a 12 hour hour dosing interval, venipuncture blood sampling is impractical for routine calculation of AUC. The simplicity of OF sample collection allows multiple sampling, therefore, estimating AUC and C_{max} will be possible. A few reports were published on immunosuppressant, namely, cyclosporine A [19, 29], mycophenolic acid [17, 30, 31] and TAC [20].

The aim of this study was to study factors that may affect correlation between the TAC concentrations in blood and OF, as well as the quality of OF samples obtained at different sampling condition.

Study population

Studies protocols approved by Institutional Review Board of Rhode Island Hospital (Providence, RI). Samples included in this paper were collected, in two studies, from patients attending kidney transplant clinics. Recruited patients were on triple

immunosuppressants regimen included tacrolimus, prednisone, and mycophenolic acid.

Patients Samples

On the study day, patients underwent the physical examination by the physician and asked to sign the informed consent. In the first study, venous blood samples (about 4 mL) were collected in ethylenediaminetetraacetic acid (EDTA) accompanied by passive drooled rested OF samples collected in siliconized plastic cups. Samples collected intermittently at certain time points, including pre-dose (time 0 = C_0). In the second study, blood samples were collected at C_0 and C_2. Matching OF samples collected with ± 5 min from blood sample time at resting, 5 min after mouth rinsing using bottled water, and instantly after taking a saliva stimulant (patients asked to put a commercial sour candy in their mouth for 10 second with continues tong movement). After pre-dose samples collection, patients were given a voucher for free breakfast and asked to report back at study location shortly before C_2 sampling time when blood and corresponding OF samples were collected. All blood and OF samples kept on dry ice till transferred to the Biomedical and Pharmaceutical Sciences (BPS) department at University of Rhode Island and stored at -80 °C till analyzed. Participant's Demographic information is showed in **Table 1.**

Measuring TAC in blood and OF

Details of the LC-MS/MS method used to quantify TAC blood concentration is described elsewhere [32]. In brief, sample extraction involved mixing 200µL of blood sample with 800µL precipitating solution of ZnSO4 (17.28 g/L): methanol (30:70,

v/v) mixture contained ascomycin as internal standard (100ng/mL). After vortex mixing, samples centrifuged for 10min at 13000 rpm.

Measuring TAC in OF

TAC concentration in OF was measured using a validated LC-MS/MS method (manuscript II). In brief, chromatography separation was achieved with a run time of 2.2 min using an Acquity UPLC BEH C18 column kept at 60 °C and. Gradient elution consisted of 2mM ammonium acetate/0.1 formic acid in water (mobile phase A) and in methanol (mobile phase B) at 0.4 mL/ min flow rate. Initial mobile phase composed of 50% solvent (B) increased gradually to 98% over 0.5 min and maintained at this level till 1.8 min. Then mobile phase returned to initial conditions within 10 seconds and maintained until the end of the run at 2.2 min to recondition the column for the next run. The elution times of TAC and internal standard were 1.0 min.

A simple sample preparation and extraction procedures were followed, involved adding 50µL of OF sample with 100µL of ACN precipitating solvent containing internal standard (ascomycin, 600 ng/L) in 1.5 mL polypropylene tube. After vortex mix for 10 seconds, the mixture was centrifuged at 10,000xg for 5 min at 20°C. The supernatant was then transferred into an auto-sampler vial, and 10 µL was injected. The dynamic range of was 30- 4800 ng/L. The lower limit of quantification (LLOQ) was set at the concentration that had a signal-to-noise ratio (S/N) of ≥10; accuracy of 80-120%; and a Coefficient of Variation (CV) less than 20%. Acceptance criteria for QCs included accuracy between 85-115% and CV less than 15%. Selectivity assessed by inspecting the presence of noise or peaks in chromatograms represent blank OF samples injections (from 6 donors) compared with LLOQ sample chromatogram.

Statistical data analysis

Statistical analysis was performed using the SPSS software (version 22, SPSS Inc., Chicago, IL, USA). Normal distribution of the data was checked graphically and confirmed with the Shapiro-Wilk test, and nonparametric tests were used whenever needed.

Genomic studies:

DNAzol kit was used to extract genomic DNA from blood samples obtained from each patient as described in manufacture's protocol (Invitrogen Corporation, Carlsbad, CA, USA). Samples genotyped for SNPs in for *CYP3A and P-glycoprotein*. The genotyping process utilized allelic discrimination with a TaqMan® Drug Metabolism Genotyping Assay. Life Technologies 7500 Real-Time PCR system (Life Technologies, Foster City, CA) was used for SNP analysis.

Salivary blood contamination

To assess and quantify possible salivary blood contamination, transferrin kit from Salimetrics LLC (State College, PA, USA) was used following manufacture's recommendation [33]. Transferrin quantification was performed using SpectraMax M5e Microplate Reader (Sunnyvale, CA, USA).

Results and discussion

12-hours profile

Eighty-five OF samples collected from 10 patients at rest. All samples had TACs within assay's validated range (30-4800 pg/mL). The concentration of TACs and blood were 5.57±2.58 and 863±641, respectively

2 hours profile study

In total, 184 OF samples were analyzed. Five samples were excluded (four samples had TAC concentration less than LLOQ all of them were stimulated samples, and one rested C_0 sample had visible blood contamination). On the other hand, 4 OF samples had TAC concentration higher than upper limit of quantification; all of them had salivary transferrin (TRNs) level higher than transferrin salivary assay quantification range (0.08 - 6.6 mg/dL). Four additional samples had TRNs higher than the upper limit of quantification. In total 171 samples were eligible for further data analysis. Following manufacturer's recommended threshold of ≤1mg/dL TRNs, 131samples would be eligible for further analysis (**Table 2)**. Samples with TRNs level >1mg/dL were 14 rested, 11 rinsed and 4 stimulated samples collected at C_0 and 7 rested, 2 rinsed and 2 stimulated samples collected at C_2.

Blood contamination and TACs concentration

Transferrin is a plasma protein with molecular weight of 76000 [33]. The presence of TRN in OF is an indication of injury in oral cavity [33]. The possibility of salivary blood contamination may increase in the presence of micro injuries from poor oral hygiene, some infectious diseases and smoking [33]. TRNs level showed to have diurnal variation, with a higher level in the afternoon compared with earlier collected samples [34]. Contradicting finding regarding the effect of gender differences in the TRNs level in children (higher in boys) [34] and adults (higher in females) [35]. Therefore, using TRNs as biomarker of salivary blood contamination should take into the consideration the physiological and environmental factors that may alter the TRNs level. About 85% of tacrolimus distribute into red blood cells [36]. Therefore, the presence of blood traces in OF fluids may compromise the integrity of results. The

association between the amount of TRNs and TACs in samples collected at different sampling conditions was investigated to determine samples to be included if final data analysis.

The effect of increasing salivary TRN level seems to be compound specific. The minimum amount of TRN in oral fluids showed a significant increase in testosterone concentration, but for cortisol, the association only seen if signs of visual discoloration are seen [37]. The concentration of mycophenolic acid in the oral fluid also showed an elevated level accompanied by increased level of TRNs (excluding samples with TRNs >1 mg/dL), in pre-dose fasted samples [17]. In this study, we measured the transferrin concentration in oral fluid as a biomarker for blood contamination to determine the threshold value at which TACs measurements would be compromised.

Following manufacturer's recommended threshold of ≤1mg/dL TRNs, data analysis revealed a significant correlation between TACs and TRNs concentration (p-values <0.05) in some sampling condition (**Table 2,** R-values denoted with *****).

High TRNs is an indication of salivary blood contamination that may artificially overestimate drugs concentration in OF [34]. Assumed increases of TACs levels in response to high TRNs level was investigated in all sub-groups by calculating mean+1STD and mean+2STD of TRNs in all samples with TRN level within the dynamic range of the assay ≤6.6mg/dL (**Table 3)**. All samples that have TRNs level falls within mean+1STD or mean+2STD were included, and the correlation between produced values (mean+1STD or mean+2STD) and TACs was tested. As can be seen from **Table 4**, strong correlation in the same subgroups still exist (P value <0.5) despite changes in TRNs levels. In addition, when all samples with TRNs ≤6.6mg/dL

included, statistically insignificant correlation is seen (**tables 5**) which is comparable to those when only samples with TRNs ≤1mg/dL included (**Tables 2**). Furthermore, when 20 samples with highest TRNs level (**Table 6**) plot against TACs, no correlation is seen (**Figures 1)**. These results are agreement with previous study [20]. In this study, the effect of blood contamination on TACs level was investigated by spiking OF with increasing amount of blood contained 11.2 μg/L TAC. Only samples that showed signs of discoloration had elevated TAC level between 4.5 and 28%. Given all above, it seems that there is insignificant/weak correlation between TACs and TRNs concentration in samples with TRNs ≤6.6mg/dL; therefore all samples with TRNs ≤ 6.6mg/dL were included in further data analysis.

Effect of different sampling conditions

Changing salivary flow rate may alter the drug concentration drug concentrations in the OF via altering contact time and the pH, consequently, affecting tubular re-absorption and secretion [21, 38]. Changes of flow rate may affect some drugs but has little to no effect on others [21, 38]. Tacrolimus is a highly lipophilic compound with logP and pka value of 3.19- 5.59 and 9.96, respectively [39]. These characteristics make TAC non-ionized in physiological pH, therefore, ideal for OF therapeutic drug monitoring. Additionally, food consumption produces protein-rich OF compared with protein-poor OF produced from other stimuli [40]. In this study, the effect of different sampling conditions on quality of OF samples, as determined by TRNs level, and the correlation between TAC concentrations in OF and blood, were studied.

The concentrations of TAC in OF in all subgroups are showed in **Figures 2A** and **2B.** In both time points, there is as decrease in concentration, with the highest level in the rested sample followed by rinsed and stimulated samples.

Correlation between TAC in OF and blood

Interestingly, when comparing same sampling conditions across the two time points, the mean concentration of TACs in C_2 samples were always lower compared to C_0 samples (**Figures 3A**, **3B** and **3C,** not statistically significant), despite the fact of significantly higher TAC in C_2 blood samples (**Figures 3D**). We attribute this to the possible effect of food as C_2 samples were collected after serving the breakfast. Correlation between salivary and blood TAC concentrations are presented in **Figures 4A** and **4B**. As can be seen, the correlation was best in rinsed samples collected at C_0; therefore, this subset of data was selected to check possible covariate effect.

Metabolizing enzymes CYP3A4 [41] and P-gp transporter are expressed in minor and major salivary glands [42, 43]. The possible effect of genetic polymorphism in CYPA3 enzymes and P-gp on the association between TAC concentration in OF and blood was examined. Nonetheless, no statistically significant differences were seen in different genotyped patients.

12 hours profile study

The High correlation between TACs and TRNs was seen in samples collected over 12 hours period (**Figure 5A**, R= 0.67, p <0.001). However, weak correlation was seen between TAC concentration in OF and blood (**Figure 5B,** R = 0.13, p= 0.21). These results are online with these obtained in 2 hours profile study in which poor correlation in TAC concentration in OF and blood was seen.

Conclusion

Using OF as an alternative to blood for TDM is appealing due to ease and low-cost of sampling. Many factors may alter drugs levels in OF. Results of this study indicate that salivary blood contamination has minimal on TACs when TRN level was ≤ 6.6 mg/dL. Better correlation between oral fluid and blood tacrolimus concentration was observed in fasted samples collected at pre-dose as compared with non-fasted samples collected post dose.

Table 3-1: Summary of demographic information of participants

	Mean±STD	Range
Gender M/F	27/19	-
Age (years)	40.2 ±14.6	21-74
Hight	168.9 ±10.3	155.0-185.4
Weight (kg)	90.4±22.6	52.7-122.5
Total Albumin (mg/mL)	35.3 ±3.5	29.6-40.4
Creatinin clearance	77.8±22.0	56.6-127.2
Tacrolimus Concentration	7.7±4.0	1.7-21.1
A1C		
Diabetic	6.0±0.97	6.4-11.7
Non-diabetic	8.4±1.7	4.4-8.9
Genotyping	Wild type	Mutant type
ABCB1.3435C.T	15	31
ABCB1.2677G>T/A	14	32
ABCB1.1236C>T	21	25
CYP3A4.In6.C>T	42	4
CYP3A4..285A>G	39	7
CYP3A5.219.237A>G	11	35

Table 3-2: Correlation between salivary tacrolimus and transferrin concentrations (≤1 mg/dL).

Sampling Time (hrs)	**Sampling conditions**	**N**	**R**
0	Rested	12	0.234
	Rinsed	18	0.195
	Stimulated	25	0.701*
2	Rested	19	-0.371
	Rinsed	29	0.422*
	Stimulated	28	-0.105
	Total	131	

*P value < 0.05

Table 3-3: shows mean, mean+1std and mean+2std transferrin concentration of all samples transferrin level ≤6.6 mg/dL and TACs

Time (hrs)	Sampling conditions	N	mean	std	mean+1std	mean+2std
	Rested	26	1.55	1.50	3.05	4.55
	Rinsed	29	0.89	0.54	1.43	1.97
0	Stimulated	29	0.42	0.39	0.81	1.19
	Rested	26	0.83	1.24	2.07	3.31
	Rinsed	31	0.42	0.34	0.77	1.11
2	Stimulated	30	0.43	0.40	0.83	1.23
	Total	171				

Table 3-4: Correlation between TACs and TRNs including samples that have transferrin level of mean+ 1 std or mean+ 2 std.

Sampling time (hrs)	**Sampling conditions**	**mean+1std**		**mean+2std**	
		N	**R**	**N**	**R**
0	Rested	23	0.241	24	0.335
	Rinsed	24	0.203	28	0.263
	Stimulated	24	0.585*	27	0.671*
2	Rested	23	0.337	25	0.617*
	Rinsed	23	0.322	29	0.422*
	Stimulated	24	0.146	29	0.126
	Total	141		162	

*P value < 0.05

Table 3-5: Correlation between salivary transferrin (<6.6 mg/dL) and concentration.

Sampling Time (hrs)	Correlation between TRN (<6.6) and TACs Sampling conditions	N	R
0	Rested	26	0.074
	Rinsed	29	0.225
	Stimulated	29	0.559*
2	Rested	26	0.751*
	Rinsed	31	0.439*
	Stimulated	30	0.085
	Total	171	

*P value < 0.05

Table 3-6: Oral fluid samples with highest transferrin concentration

Nominal time (hrs)	**Sampling conditions**	**Tacrolimus concentration in oral fluids**	**Transferrin level in oral fluids ≤6.6mg/dL**
0	Rested	919	6.5
2	Rested	3127	5.9
0	Rested	1040	5.4
0	Rested	2143	3.5
2	Rested	2392	2.5
2	Rested	1786	2.5
0	Rested	2425	2.4
0	Rested	500	2.3
0	Rinsed	664	2.2
0	Rested	378	2.2
0	Rinsed	487	1.9
0	Rinsed	784	1.7
0	Rinsed	365	1.7
0	Rinsed	2538	1.6
0	Rested	1793	1.6
0	Rested	1297	1.5
2	Stimulated	877	1.4
0	Rested	2609	1.4
0	Rested	1907	1.4
2	Rested	1571	1.4

Figure 3-1: Correlation between transferrin and tacrolimus in oral fluids in 20 samples with highest transferrin concentration.

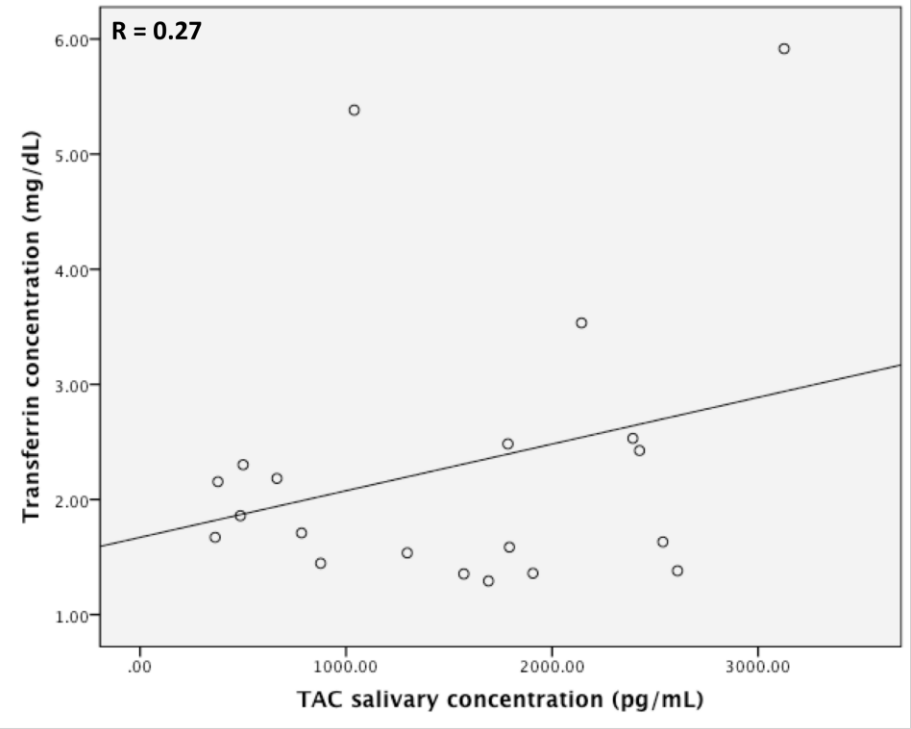
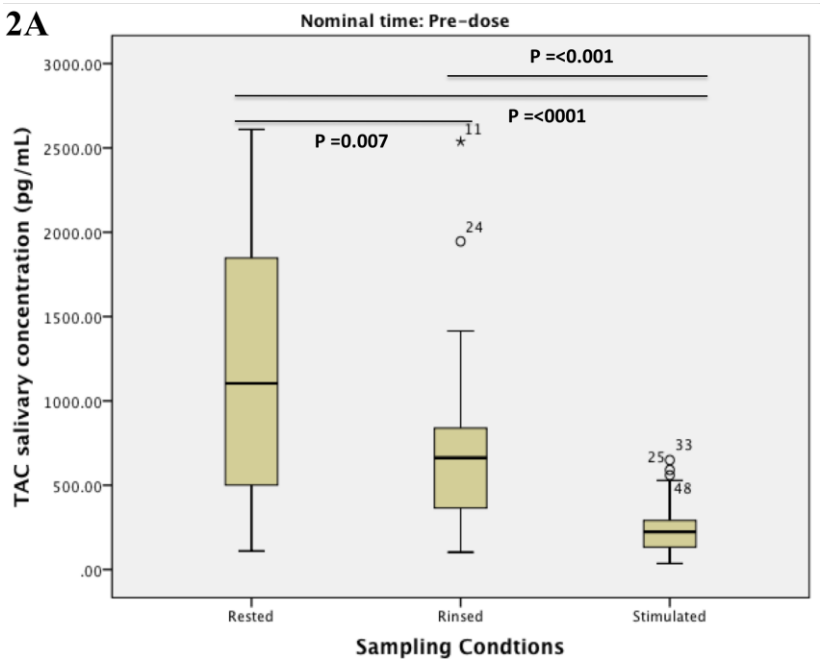


Figure 3-2: levels of tacrolimus at different sampling conditions in pre-dose samples (2A) and post-dose samples (2B).



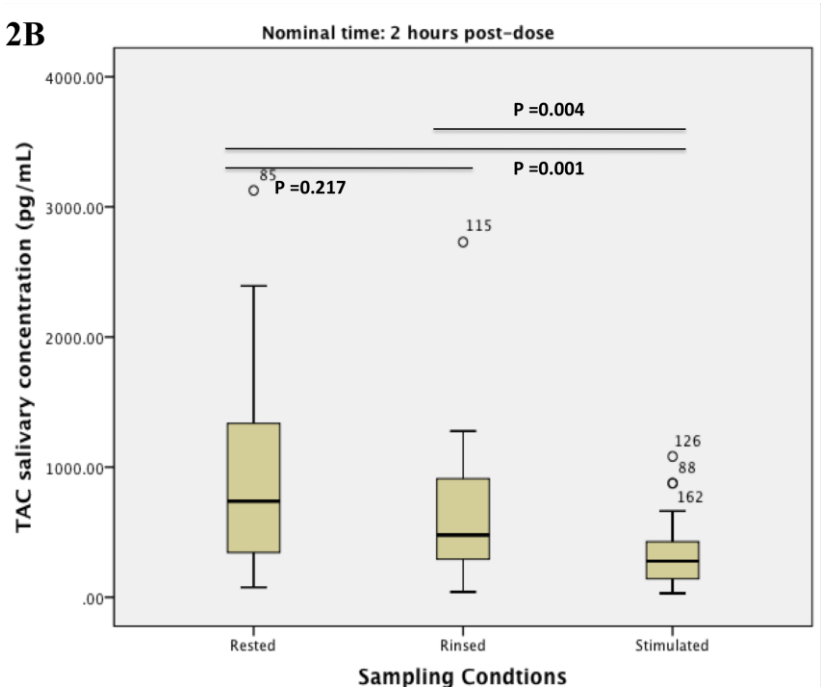


Figure 3-3: plots compare tacrolimus levels in oral fluids samples collected at rest (3A), after mouth rinse (3B), stimulated samples (3C), and in blood samples (3D) collected at pre and post dose. As can be seen, salivary levels of tacrolimus tend to be lower in 2 hours post dose oral fluid samples despite higher level in the corresponding blood samples.

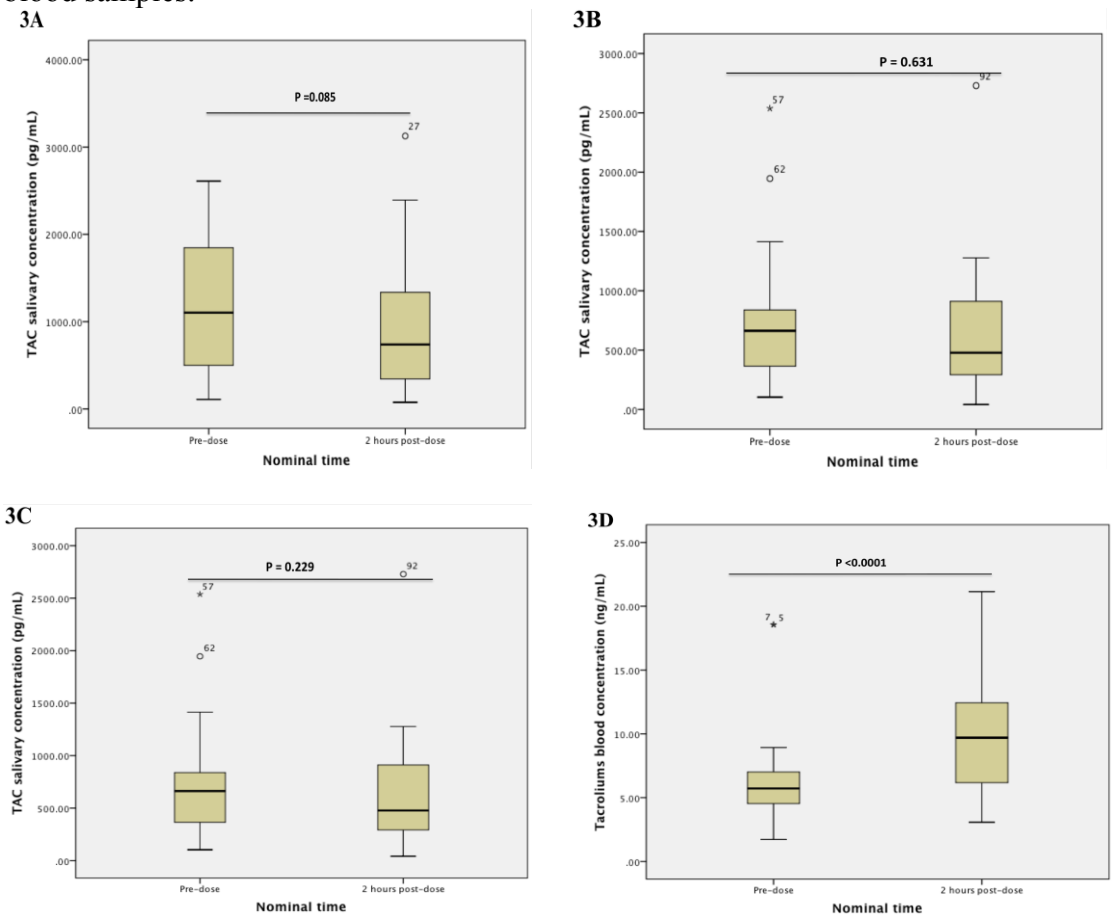
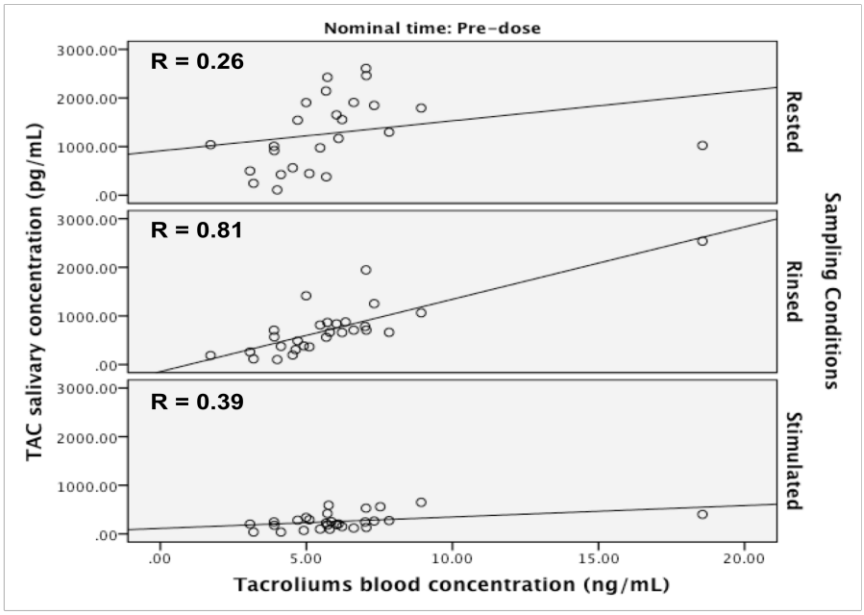


Figure 3-4: plots show the correlation between tacrolimus level in oral fluids and blood at different sampling conditions in pre-dose samples (4A) and 2 hours post dose samples (4B).

4A



4B

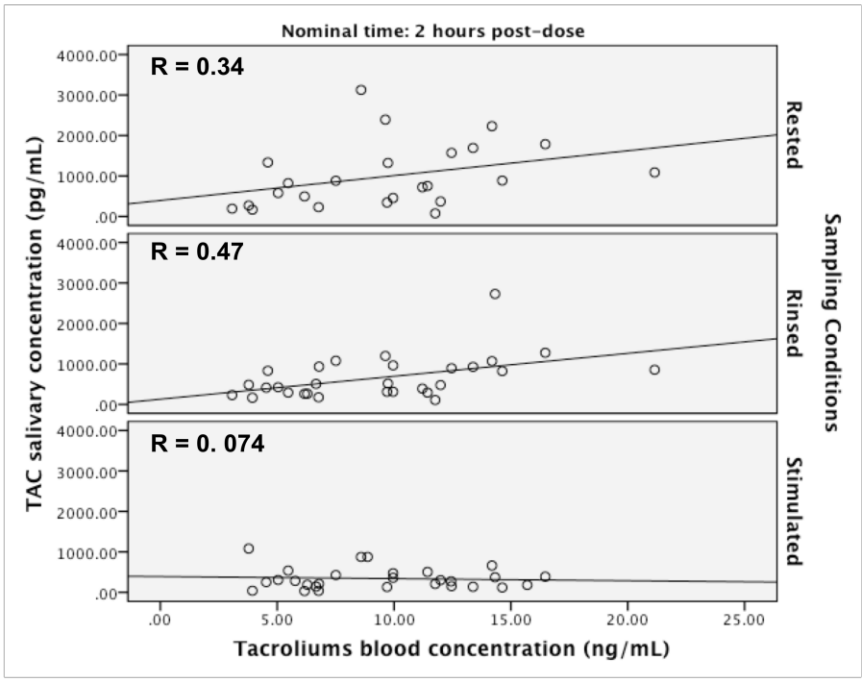
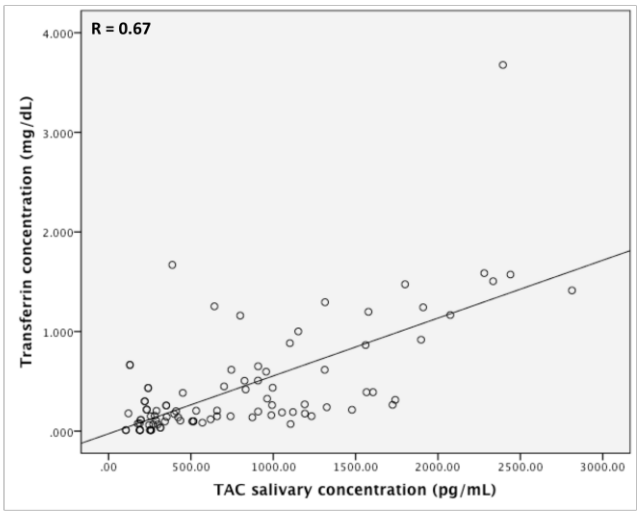
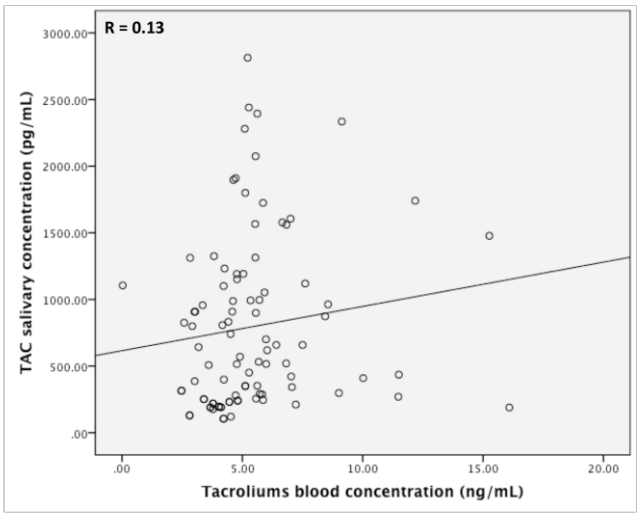


Figure 5: plots data from 12 hours profile study show the correlation between salivary tacrolimus and transferrin concentrations (5A) and tacrolimus concentrations in oral fluids and blood (5B)

5A



5B



References

1. Mohammadpour, N., et al., *A review on therapeutic drug monitoring of immunosuppressant drugs*. Iran J Basic Med Sci, 2011. **14**(6): p. 485-98.

2. Thomson, A.W., C.A. Bonham, and A. Zeevi, *Mode of action of tacrolimus (FK506): molecular and cellular mechanisms*. Ther Drug Monit, 1995. **17**(6): p. 584-91.

3. Halloran, P.F., *Immunosuppressive drugs for kidney transplantation*. N Engl J Med, 2004. **351**(26): p. 2715-29.

4. Iwasaki, K., *Metabolism of tacrolimus (FK506) and recent topics in clinical pharmacokinetics*. Drug Metab Pharmacokinet, 2007. **22**(5): p. 328-35.

5. Staatz, C.E., L.K. Goodman, and S.E. Tett, *Effect of CYP3A and ABCB1 single nucleotide polymorphisms on the pharmacokinetics and pharmacodynamics of calcineurin inhibitors: Part I*. Clin Pharmacokinet, 2010. **49**(3): p. 141-75.

6. Ro, H., et al., *Impact of tacrolimus intraindividual variability and CYP3A5 genetic polymorphism on acute rejection in kidney transplantation*. Ther Drug Monit, 2012. **34**(6): p. 680-5.

7. Shi, Y., et al., *Influence of CYP3A4, CYP3A5 and MDR-1 polymorphisms on tacrolimus pharmacokinetics and early renal dysfunction in liver transplant recipients*. Gene, 2013. **512**(2): p. 226-31.

8. Uesugi, M., et al., *Effect of intestinal CYP3A5 on postoperative tacrolimus trough levels in living-donor liver transplant recipients*. Pharmacogenet Genomics, 2006. **16**(2): p. 119-27.

9. Blume, H., et al., *Pharmacokinetic drug interaction profiles of proton pump inhibitors*. Drug Saf, 2006. **29**(9): p. 769-84.

10. Hooper, D.K., et al., *Risk of tacrolimus toxicity in CYP3A5 nonexpressors treated with intravenous nicardipine after kidney transplantation*. Transplantation, 2012. **93**(8): p. 806-12.

11. Maguire, M., T. Franz, and D.S. Hains, *A clinically significant interaction between tacrolimus and multiple proton pump inhibitors in a kidney transplant recipient*. Pediatr Transplant, 2012. **16**(6): p. E217-20.

12. Takahashi, K., et al., *Lansoprazole-tacrolimus interaction in Japanese transplant recipient with CYP2C19 polymorphism*. Ann Pharmacother, 2004. **38**(5): p. 791-4.

13. Fukudo, M., et al., *Impact of MDR1 and CYP3A5 on the oral clearance of tacrolimus and tacrolimus-related renal dysfunction in adult living-donor liver transplant patients*. Pharmacogenet Genomics, 2008. **18**(5): p. 413-23.

14. Capron, A., et al., *CYP3A5 and ABCB1 polymorphisms influence tacrolimus concentrations in peripheral blood mononuclear cells after renal transplantation*. Pharmacogenomics, 2010. **11**(5): p. 703-14.

15. Staatz, C.E. and S.E. Tett, *Clinical pharmacokinetics and pharmacodynamics of tacrolimus in solid organ transplantation*. Clin Pharmacokinet, 2004. **43**(10): p. 623-53.

16. Cheung, C.Y., et al., *Dried blood spot measurement: application in tacrolimus monitoring using limited sampling strategy and abbreviated AUC estimation*. Transpl Int, 2008. **21**(2): p. 140-5.

17. Mendonza, A.E., R.Y. Gohh, and F. Akhlaghi, *Analysis of mycophenolic acid in saliva using liquid chromatography tandem mass spectrometry*. Ther Drug Monit, 2006. **28**(3): p. 402-6.

18. Teeninga, N., et al., *Monitoring prednisolone and prednisone in saliva: a population pharmacokinetic approach in healthy volunteers*. Ther Drug Monit, 2013. **35**(4): p. 485-92.

19. Mendonza, A., R. Gohh, and F. Akhlaghi, *Determination of cyclosporine in saliva using liquid chromatography-tandem mass spectrometry*. Ther Drug Monit, 2004. **26**(5): p. 569-75.

20. Belostotsky, V., et al., *Measurement of saliva tacrolimus levels in pediatric renal transplant recipients*. Pediatr Nephrol, 2011. **26**(1): p. 133-8.

21. Feller, K. and G. le Petit, *On the distribution of drugs in saliva and blood plasma*. Int J Clin Pharmacol Biopharm, 1977. **15**(10): p. 468-9.

22. Horning, M.G., et al., *Use of saliva in therapeutic drug monitoring*. Clin Chem, 1977. **23**(2 PT. 1): p. 157-64.

23. Mucklow, J.C., et al., *Drug concentration in saliva*. Clin Pharmacol Ther, 1978. **24**(5): p. 563-70.

24. Tennison, M., et al., *Feasibility and acceptance of salivary monitoring of antiepileptic drugs via the US Postal Service*. Ther Drug Monit, 2004. **26**(3): p. 295-9.

25. Gorodischer, R., et al., *Saliva versus blood sampling for therapeutic drug monitoring in children: patient and parental preferences and an economic analysis*. Ther Drug Monit, 1994. **16**(5): p. 437-43.

26. Haeckel, R., *Factors influencing the saliva/plasma ratio of drugs*. Ann N Y Acad Sci, 1993. **694**: p. 128-42.

27. Zahir, H., et al., *Changes in tacrolimus distribution in blood and plasma protein binding following liver transplantation*. Ther Drug Monit, 2004. **26**(5): p. 506-15.

28. Reece, P.A., et al., *Prednisolone protein binding in renal transplant patients*. Br J Clin Pharmacol, 1985. **20**(2): p. 159-62.

29. Coates, J.E., S.F. Lam, and W.T. McGaw, *Radioimmunoassay of salivary cyclosporine with use of 125I-labeled cyclosporine*. Clin Chem, 1988. **34**(8): p. 1545-51.

30. Shen, B., et al., *Determination of total, free and saliva mycophenolic acid with a LC-MS/MS method: application to pharmacokinetic study in healthy volunteers and renal transplant patients*. J Pharm Biomed Anal, 2009. **50**(3): p. 515-21.

31. Wiesen, M.H., et al., *Liquid chromatography-tandem mass spectrometry method for the quantification of mycophenolic acid and its phenolic glucuronide in saliva and plasma using a standardized saliva collection device*. J Chromatogr A, 2012. **1241**: p. 52-9.

32. Chitnis, S.D., et al., *Concentration of tacrolimus and major metabolites in kidney transplant recipients as a function of diabetes mellitus and cytochrome P450 3A gene polymorphism*. Xenobiotica, 2013.

33. Schwartz, E.B. and D.A. Granger, *Transferrin enzyme immunoassay for quantitative monitoring of blood contamination in saliva*. Clin Chem, 2004. **50**(3): p. 654-6.

34. Granger, D.A., et al., *Blood contamination in children's saliva: prevalence, stability, and impact on the measurement of salivary cortisol, testosterone, and dehydroepiandrosterone*. Psychoneuroendocrinology, 2007. **32**(6): p. 724-33.

35. Fleissig, Y., et al., *Comparative proteomic analysis of human oral fluids according to gender and age*. Oral Dis, 2010. **16**(8): p. 831-8.

36. Zahir, H., et al., *Validation of methods to study the distribution and protein binding of tacrolimus in human blood*. J Pharmacol Toxicol Methods, 2001. **46**(1): p. 27-35.

37. Kivlighan, K.T., et al., *Quantifying blood leakage into the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva*. Horm Behav, 2004. **46**(1): p. 39-46.

38. Liu, H. and M.R. Delgado, *Therapeutic drug concentration monitoring using saliva samples. Focus on anticonvulsants*. Clin Pharmacokinet, 1999. **36**(6): p. 453-70.

39. DrugBank. *Tacrolimus*. [cited on 11/02/2014; Available from: http://www.drugbank.ca/drugs/DB00864.

40. Gorodischer, R. and G. Koren, *Salivary excretion of drugs in children: theoretical and practical issues in therapeutic drug monitoring*. Dev Pharmacol Ther, 1992. **19**(4): p. 161-77.

41. Kragelund, C., et al., *Expression of two drug-metabolizing cytochrome P450-enzymes in human salivary glands*. Oral Dis, 2008. **14**(6): p. 533-40.

42. Uematsu, T., et al., *P-glycoprotein expression in human major and minor salivary glands*. Arch Oral Biol, 2001. **46**(6): p. 521-7.

43. Uematsu, T., et al., *Expression of ATP-binding cassette transporter in human salivary ducts*. Arch Oral Biol, 2003. **48**(1): p. 87-90.

Chapter 4 : MANUSCRIPT IV

To be submitted to Clinical Pharmacokinetics

Development and Validation of a Sensitive and Selective LC-MS/MS Method for Quantification of Mycophenolic Acid and its Glucuronide Metabolites in Oral Fluid, Plasma and Plasma Ultrafiltrate in Kidney Transplant Recipients

Mwlod Ghareeb[1], Reginald Y. Gohh [2] , Fatemeh Akhlaghi[1]

DEPARTMENT AND INSTITUTION

[1]Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, RI
[2] Division of Organ Transplantation, Rhode Island Hospital, Warren Alpert Medical School of Brown University, Providence, Rhode Island, USA.

Address correspondence to: Fatemeh Akhlaghi, PharmD, PhD, Clinical Pharmacokinetics Research Laboratory, Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, 495A College of Pharmacy, 7 Greenhouse Road, Kingston, RI 02881, USA. Phone: (401) 874 9205. Fax: (401) 874 5787. Email: fatemeh@uri.edu

DISCLOSURE

No conflict of interest is declared

ABBREVIATIONS

ACN: Acetonitrile, LC-MS/MS: Liquid chromatography tandem mass spectrometry, LLOQ: Lower limit of quantification ME: Matrix effect, MeOH: Methanol, MPA: Mycophenolic acid, MPAf: Unbound mycophenolic acid concentration in plasma, MPAof: Oral fluid mycophenolic acid concentration, MPAt: Total mycophenolic acid concentration in plasma, MPAG: Mycophenolic acid glucuronide, MPAf: Unbound mycophenolic acid glucuronide concentration in plasma, MPAof: Oral fluid mycophenolic acid glucuronide concentration, MPAt: Total mycophenolic acid glucuronide concentration in plasma, OF: Oral fluid, PLs: Phospholipids

Abstract

Free drug fraction in the blood is responsible for pharmacological effect and toxicity. However, quantifying unbound fraction is costly and labor intensive. Drug fraction in oral fluid (OF) is believed to be in equilibrium with plasma free fraction. Therefore, OF may provide a mean for estimating unbound fraction in noninvasively collected samples and with a simple sample preparation procedure. In this manuscript, a liquid chromatography tandem mass spectrometry method was developed and validated and used to quantify the concentration of mycophenolic acid (MPA) and its glucuronide metabolites (MPAG) in oral fluid, plasma and in plasma ultrafiltrate. A simple, sensitive and selective method was developed for quantification of salivary, unbound, total MPA and MPAG. The robustness of the method was confirmed by incurred sample reanalysis test. The method was successfully used for quantifying the analytes in samples obtained from stable renal transplant recipients.

Introduction

Mycophenolic acid (MPA) is an immunosuppressive agent that is widely used in solid organ transplantation. In United States, in year 2005, about 87% of kidney and pancreas transplant patients were prescribed MPA at hospital discharge [1]. It is metabolized by uridine diphosphate glucuronosyltransferases (UGTs) to the major inactive metabolites mycophenolic acid β-D-glucuronide (MPAG) and the minor but pharmacologically active metabolites mycophenolic acid acyl-β-D-glucuronide (AcMPAG) [2]. MPA highly binds to plasma protein with only 1-3% found in free form [2]. In patients with compromised renal, MPAG metabolites level may increases by 3-6 folds, resulting displacement of MPA from plasma protein binding sites [2]. As

a result, MPA free fraction may increase up to 7% [2]. Currently, whole blood or plasma obtained through venipuncture is used for TDM of immunosuppressive agents [3]. Because of the invasive nature of blood sampling, alternative matrices were investigated, including OF [4-6] and dried blood spot [7]. Because of the narrow therapeutic index, therapeutic drug monitoring of MPA is recommended.

Few reports were previously published utilizing liquid chromatography tandem mass spectrometry (LC-MS/MS) for quantification MPA in OF (MPAof) [6]; MPAof, plasma free fraction (MPAf), and total plasma (MPAt) [5]; and total MPAt, MPAof, total glucuronide metabolites (MPAGt), and oral fluid MPAG metabolites (MPAGof) concentrations [4]. None of these methods, however, has quantified MPA and MPAG in OF, concurrently with free and total MPA and MPAG in plasma. In this paper, a simple, sensitive and robust LC-MS/MS method was developed for quantification of MPA and MPAG in OF, as well as, their free and total plasma concentrations. In this method, a simple samples preparation procedures were employed using liquid-liquid extraction with acetonitrile (ACN) with good recovery, is presented. The quality of the method was assessed by re-measurement some of the randomly selected patient samples (Incurred Samples Reanalysis procedure) obtained from renal transplant recipients.

Chemicals and reagents

Mycophenolic acid and ($C_{17}H_{20}O_6$, MW = 320), MPAG ($C_{23}H_{28}O_{12}$, MW= 496) and the deuterated mycophenolic acid internal standard (MPA-d3) ($C_{17}H_{17}D_3O_6$, Mw =323), in powder form were purchased from Toronto Research Chemicals (Toronto, Canada). Optima™ LC/MS grade acetonitrile (ACN), ammonium acetate

(Crystalline), and formic acid, were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was obtained using Milli-Q Synthesis system fitted with Q-Gard 2 Purification Pack (Millipore, Bedford, MA, USA). AquaSil Siliconizing Fluid was purchased from Thermo Fisher Scientific Inc. (Franklin, MA, USA). Drug-free human OF from six donors was obtained from Bioreclamation Inc. (Westbury, NY, USA). For salivary blood contamination, transferrin assay kit from Salimetrics LLC (State College, PA, USA) was used [8] and quantified with SpectraMax M5e Microplate Reader (Sunnyvale, CA, USA).

Apparatus

Oral fluid samples were sonicated using Branson® Sonicator (Danbury, CT, USA) to produce a homogeneous mixture. The supernatant was obtained from OF and plasma samples using Eppendorf 5810 centrifuge from Micro and Nanotechnology (Urbana, IL, USA). Free MPA and MPAG concentrations were measured in plasma ultra-filtrate obtained using Centrifree® Ultrafiltration device from EMD Millipore, Merck KGaA (Darmstadt, Germany).

Samples were analyzed using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). The LC-MS/MS system was consisted of Acquity UPLC from Waters Corp (Milford, MA, USA) connected to Xevo TQ MS mass spectrometry from Waters Corp. MassLynx™ software (ver. 4.1) was used to control the system and data acquisition, and data processed using TargetLynx™ tool. The UPLC system had a binary pump and equipped with built-in column heater. Twenty micro-litters sample loop was used to deliver 10 µL of extracted samples in a partial loop mode.

Chromatographic conditions

An Acquity UPLC BEH C18 (2.1 mm x 50 mm) column with a 1.7µm-particle size and 130Å pores size was used (Waters Corp) for chromatographic separation. The temperature of the column was kept at 55°C, and the auto-sampler temperature was maintained at 20°C. Gradient elution was employed with a mobile phase consisted of water containing 95:5% water: ACN mixture contained 2 mM ammonium acetate / 0.1% (v/v) formic acid (Solvent A); and ACN containing 2 mM ammonium acetate / 0.1% (v/v) formic acid (Solvent B). The mobile phase was delivered at 0.350 mL/min flow rate. The run cycle started at 85% solvent (A) slowly decreased 5% over 1.4 min and maintained at this level till 2.2 min. To re-equilibrate the column for the next run, solvent (A) was increased within 0.1 min to 85% and kept till the end of the run at 2.8 min. Diversion valve was set to deliver the first 0.65 min and from 1.60 min till the end of each run to waste. The elution times were 0.93 min MPA and MPA-d3; and 1.31 min for MPAG.

Mass spectrometry conditions

Mass spectrometry detection and quantification of MPA and MPAG performed in positive electrospray ionization (ESI) and multiple reaction monitoring (MRM) modes. Intellistart tool was used to obtain initial mass spectrometry parameters in unit mass resolution analysis mode followed by manual tuning to achieve highest possible sensitivity. Final mass spectrometry parameters were as following: collision voltage (V)= 25, 14 and 23 for MPA, MPAG and IS respectively, cone voltage (CV) = 30,

collision energy (CE) = 20 and capillary voltage (kV = 1.5), source temperature (°C) = 150, dissolution temperature (°C) = 500, cone gas flow (L/hr) = 25, desolvation gas flow (L/hr) = 1000, and collision gas flow (mL/min) = 0.15. Ammonium adducts $[M+NH_4]^+$ were selected as precursors for MRM with transitions (m/z, Q1 > Q3) of (m/z, 338.41>207.28) (m/z, 514.54 > 207.26), and (m/z, 341.45 > 210.33) for MPA, MPAG and MPA-d3 respectively.

Preparation of solution for standard, quality control and internal standard

Stock, sub-stock and working stock solutions of MPA and MPAG were prepared by reconstitution in 80% ACN. Internal standard stock and working solution were prepared in 100% ACN. All solutions were stored at – 20 °C. Standards and quality controls (QCs) were prepared by spiking the OF and plasma with serially diluted working stock solutions to achieve desired concentrations. The concentrations of internal standard working solution (precipitating solvent) were set at 0.005, 0.025 and 0.05 µg/mL for OF, ultra-filtrate and plasma samples.

Clinical Samples

Patients' samples included in this report were collected from patients attending kidney transplant clinic at Rhode Island Hospital (Providence, RI) after the hospital's Institutional Review Board approved the study protocol. About 4 mL of blood collected in ethylenediaminetetraacetic acid (EDTA) along with matching OF samples. All samples kept on dry ice till transferred to the Biomedical and

Pharmaceutical Sciences (BPS) department at University of Rhode Island and stored at – 80 °C till analyzed.

Sample extraction

Plasma and OF calibration curve standards, quality controls (QCs), blank, and patients' samples were allowed to thaw at room temperature. Oral fluids samples were vortexed for 5 seconds and sonicated for 5-10 seconds (depending on samples volume) to breakdown salivary components and produce a homogenous mixture. Fifty micro-liters of OF or whole plasma samples loaded into 96-well plates contained 100 µL and 200 µL precipitating solution in each well for OF or plasma respectively. The plate mixed on an automatic shaker for 5 min followed by centrifugation a speed of 3000 xg for 5 min at 20°C using swing rotor rack. The supernatant then transferred to a new plate using a multichannel pipette. For MPAf and MPAGf concentrations measurement, 300 µL of calibration curve standards, quality controls (QCs), blank, and patients' plasma samples were loaded into the ultra-filtration devices and centrifuged following manufacturer's recommendation. One part of ultra-filtrate was diluted with five parts of 50% ACN, briefly vortex mix and transferred to an auto-sampler 96-wells plate. The injection volume was 5 µL for OF and plasma; and 10 µL for plasma ultra-filtrate.

Statistical data analysis

Statistical analysis was performed using GraphPad Prism software.

Assay validation

Sensitivity and selectivity

The method was validation in accordance with the current version of FDA guidance for industry bioanalytical method validation [9]. The lower limit of quantification (LLOQ) was determined by the concentration that had signal/noise ratio of at least 10, accuracy between 80-120%, and Coefficient of Variation (CV) less than 20%. Acceptance criteria for QCs were accuracy between 85-115% and CV less than 15%. Selectivity assessed by inspecting the presence of noise or peaks in chromatograms represent blank OF, whole plasma and plasma ultrafiltrate samples (from 6 donors) compared with LLOQ samples chromatograms.

Stability

Stability studies were performed by measuring MPA and MPAG in each matrix, in three replicate, at QC1 and QC3 concentrations. Stability studies included freeze and thaw (after three freeze and thaw cycles), bench-top (for up to 8hrs) and auto-sampler (by re-injecting one of validation batch after it was left in the auto-sampler for 24hrs).

Matrix effect and Recovery

The presence and the possible effect of matrix effect (ME) in all matrices studied in two different ways. *In the first approach*, chromatograms obtained from post-column infusion test were inspected visually. This test involved continuous infusion of 95% ACN (which represents the composition of mobile phase at elution time of the analytes and the IS) contains MPA, MPAG and the MPA-d3 at concentrations around

highest standards point for each matrix at 10uL/min flow rate after the column through a Tee connection. After establishing the baseline, extracted blank samples OF, plasma, or ultra-filtrate was injected using pre-established LC method. The obtained chromatogram was checked for signs of ion suppression and/or enhancement in comparison to chromatograms of blank injection of neat solution (1:2, water: ACN). *In the second approach*, possible interference of matrices components, namely, the phospholipids (PLs) was studied. The MRM transitions of abundant PLs were added to the MS method to enable us visually locate their elution region.

Recovery of MPA and MPAG from OF was assessed by analyzed two sets of QCs samples (in 3 replicate). The First set was prepared by extracting blank OF first with ACN, followed by adding MPA or MPAG working stock solutions to achieve required concentrations (post-extraction spiked samples). The second set was prepared by spiking OF first with stock solutions followed by extraction with ACN (pre-extraction spiked samples). The recovery was determined by calculating percentage ratio of mean peaks area of pre-extraction samples to post-extraction spiked samples.

Results

Sensitivity and selectivity

In-source conversion of glucuronide metabolites MPAG to MPA has been reported [10], therefore full chromatographic separation is essential to avoid over estimation of MPA concentration. In fact, the in-source conversion was very obvious in MPA channel $[M + H]^+$ at m/z 321.35 > 207.27 (**Figure 1**). Given final chromatographic conditions, a full separation was achieved. Other adducts were identified for MPA included ammonium $[M + NH_4]^+$ and sodium adducts $[M + Na]^+$. The highest intensity

was seen in $[M + NH_4]^+$ adduct at m/z 338.41 >207.28. Other fragments of ammonium adduct with lower intensity were m/z 338.41 >303.47 and m/z 338.41 >321.44. For MPAG, three ammonium adducts identified which are 514>321, 514>303, and 514.54>207.26. The later transition showed the highest sensitivity, therefore, was chosen.

Chromatograms obtained from acquiring pooled blank samples of each matrix, from six subjects, were visually inspected and compared to chromatograms of blank neat solution (50% ACN) for any unusual peaks or noise at elution region. No sign of interference was noticed. No carryover was detected when blank extracted matrices were injected following highest calibration concentration. Representative chromatograms of LLOQ of MPA and MPAG in each matrix are shown in **Figure 2**. Calibration curves ranges of the anlytes were 0.001-1µg/mL and 0.004-1µg/mL in OF; 0.05-50 µg/mL and 1-100µg/mL in plasma ultrafiltrate; and 0.1-151µg/mL and 1-100 µg/mL in plasma; for MPA and MPAG, respectively. Analytes to internal standard peak ratio against nominal concentration used to construct the calibration curve and fitted using (1/x) weighting method. To determine accuracy and precision of the assay, three different batches of OF and plasma (for total and unbound concentration) were spiked with working stocks solution to achieve QCs concentrations (6 replicate) and extracted as described in *sample extraction* section. Accuracy and precision of the assay are showed in **Table 1.**

Stability

Bench top, freeze and thaw, auto-sampler, were studied (**Table 2**). No stability problems were noticed, and analytes were stable in extracted matrices for up to 24hrs.

Recovery and matrix effect

Samples processing and extraction procedures showed excellent recovery form both OF and plasma. The recovery ranged in OF and plasma from **88.71-103.09% for both MPA and MPAG (Table 2).** In a recent paper [4] MPA and its metabolites, MPAG, were quantified simultaneously with 82.1and 65.7% recovery, respectively, following solid phase extraction procedures.

Biological fluids contain endogenous components that may interfere and compete with analytes of interest at the ionization site in LC-MS/MS [11-13]. The ME is the term that describes this phenomenon. The ME may lead to either ionization suppression or enhancement, both of which may compromise the integrity of the results [11-13].

To investigate possible inference of matrices component, post-column infusion technique was utilized [13]. Figures 3A, 3B and 3C show composite chromatograms obtained by post-column infusion of MPA, MPAG, and MPA-d3, respectively, overlaid on chromatograms represents injections of blank matrices (OF, plasma, and plasma ultra-filtrate) and blank solvent using pre-established LC method, as well as chromatogram of MQC injection. Comparing traces of all three blank specimens injection with traces of blank solvent injection reveals an area of ion suppression between 0.25 and 0.7 min. There were no sign of ionization suppression or enhancement at the retention time of analytes or IS.

Additionally, ME was also investigated visually by monitoring their MRM transitions. Mass transitions of PLs included (m/z, 496 >184, 520 >184, 522>184, 524 >184, 758 >184, 782 >184) [11, 12]. **Figure 4A, 4B, and 4C** show the detected PLs and their

elution regions in OF, ultra-filtrate, and plasma, respectively. As can be seen, the investigated PLs eluted far enough after analytes of interest.

Finally, the use of plasma samples obtained from healthy volunteers in preparing calibration curve may not completely mimic plasma obtained from transplant patients. Transplant patients usually co-prescribed a large number of medications to prevent rejection and manage coexisting conditions [14, 15]. Therefore, incurred sample reanalysis test was performed by re-measure about 10% of patient's samples [16]. As can be seen in Figures 5A, 5B, and 5C, great agreements between two repeated measurements of MPA (upper) and MPAG (lower) in OF, plasma, and plasma ultra-filtrate, respectively. In these figures, Bland and Altman plots constructed by plotting the differences between paired repeated measurements against their average reveal good agreement between the two repeated measurements. All points lie between or near the 95% confident interval lines (dotted line).

Conclusion

In this paper, sensitive, selective and robust method for quantification of MPA and MPAG metabolites in OF, plasma, and plasma ultra-filtrate is presented. Simple sample preparation and extraction protocol was developed and used to provide minimum sample dilution and appropriate samples cleanliness, excellent recovery and minimum sample components interference.

Table 4-1. Summary of quality control samples from three individual runs.

		LLOQ		QC1		QC2		QC3	
		MPA	**MPAG**	**MPA**	**MPAG**	**MPA**	**MPAG**	**MPA**	**MPAG**
OF	**Nominal conc.**	**0.001**	**0.004**	**0.003**	**0.012**	**0.05**	**0.05**	**0.75**	**0.75**
	Inter-run %CV	6.35	9.53	5.37	6.72	2.97	6.20	4.73	4.82
	Inter-run %Bias	5.05	6.54	-0.76	-1.84	1.41	-6.21	1.54	5.71
Plasma ultrafiltrate	**Nominal conc.**	**0.1**	**1.0**	**0.3**	**3.0**	**2.0**	**15.0**	**11.3**	**75.5**
	Inter-run %CV	5.78	9.53	5.12	6.72	1.07	6.20	3.37	4.82
	Inter-run %Bias	4.39	6.54	-3.64	-1.84	2.57	-6.21	-3.70	5.71
Plasma	**Nominal conc.**	**0.05**	**1.00**	**0.15**	**3.00**	**4.00**	**30.00**	**37.50**	**112.5**
	Inter-run %CV	8.84	5.99	6.04	6.21	2.91	5.87	2.73	3.78
	Inter-run %Bias	5.44	-11.08	8.22	-2.51	-7.53	6.35	-5.59	-0.95

n= 18 (6 replicates for each validation run). % Relative error (%RE) 100 × (mean-nominal)/nominal, %CV=100 × std dev/mean.

Table 4-2. Results of stability studies and recovery

Table 2. Results of stability studies

Matrix	Analyte	QCs (ug/mL)		Bench top	Freeze & thaw	Auto-sampler 24 hrs	Recovery
OF	MPA	QC1	CV	1.05	4.10	2.96	99.01
			Bias %	4.77	-7.36	3.63	
		QC3	CV	1.47	3.98	2.41	99.44
			Bias %	5.37	2.51	5.72	
	MPAG	QC1	CV	2.75	8.81	6.30	103.09
			Bias %	-10.94	-2.95	-5.34	
		QC3	CV	6.30	3.33	3.10	98.04
			Bias %	-5.34	8.91	5.58	
Plasma Ultrafiltrate	MPA	QC1	CV	1.95	3.38	4.47	NA
			Bias %	6.97	4.12	0.09	
		QC3	CV	2.02	2.06	2.28	
			Bias %	5.82	3.67	6.03	
	MPAG	QC1	CV	8.40	5.92	5.92	NA
			Bias %	-3.89	3.46	3.46	
		QC3	CV	1.27	3.60	3.10	
			Bias %	8.12	7.91	5.58	
Plasma	MPA	QC1	CV	3.84	6.64	0.67	100.40
			Bias %	-11.07	-8.36	2.51	
		QC3	CV	1.41	0.47	-2.70	
			Bias %	-9.85	-12.71	2.64	
	MPAG	QC1	CV	5.79	4.97	1.56	88.71
			Bias %	-11.41	-9.04	3.77	
		QC3	CV	0.70	1.16	2.03	
			Bias %	-9.44	-2.70	3.33	

n = 3, %CV =100 × std dev/mean, % Relative error (%RE) 100 × (mean-nominal)/nominal
NA = not available

Figure 4-1: Representative chromatograms of $[M + NH]^+$ MPAG at m/z 514.54>207.26 (A); MPA $[M + NH]^+$ at m/z 338.41>207.28) (B); and MPA $[M + H]^+$ at m/z 321.53 > 207.27 (C). As can be seen in (C), there is an MPA peaks in MPA channel (m/z 321.53 > 207.27) at the retention time of MAPG as a result of in source conversion. The in source conversion is not obvious in MPA channel with m/z 338.41>207.28 transition

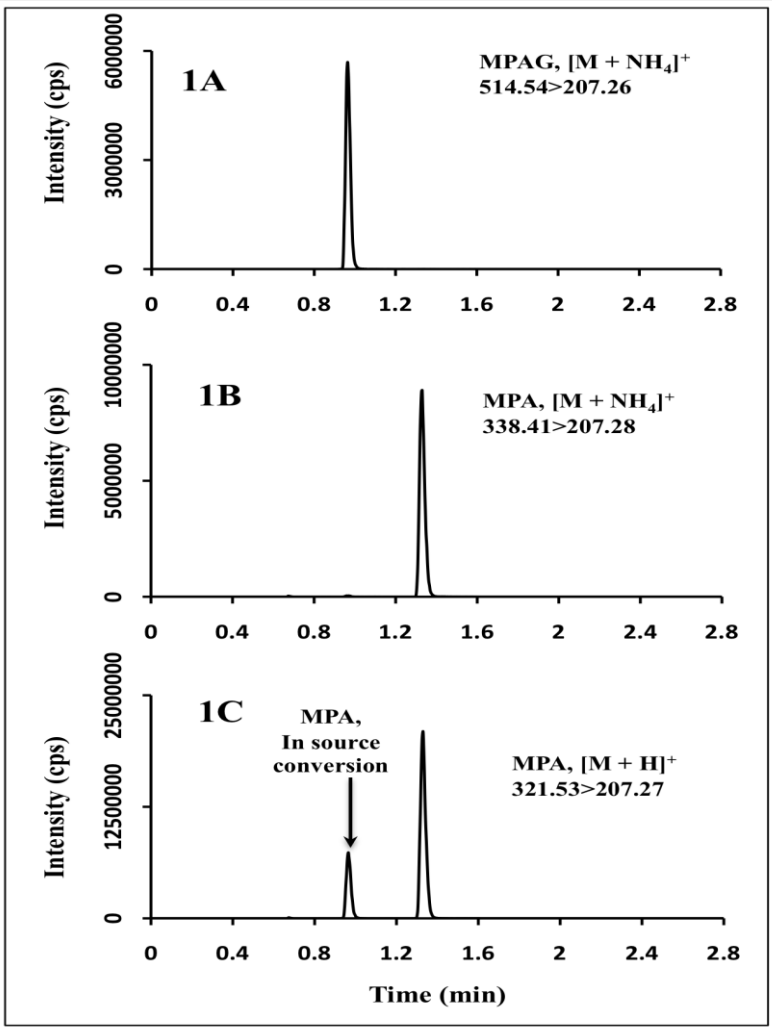


Figure 4-2: Representative chromatograms show LLOQS of MPA (2A, 2 and 2CB) and MPAG (2D, 2E and 2F) in oral fluids plasma ultrafiltrate and plasma, respectively.

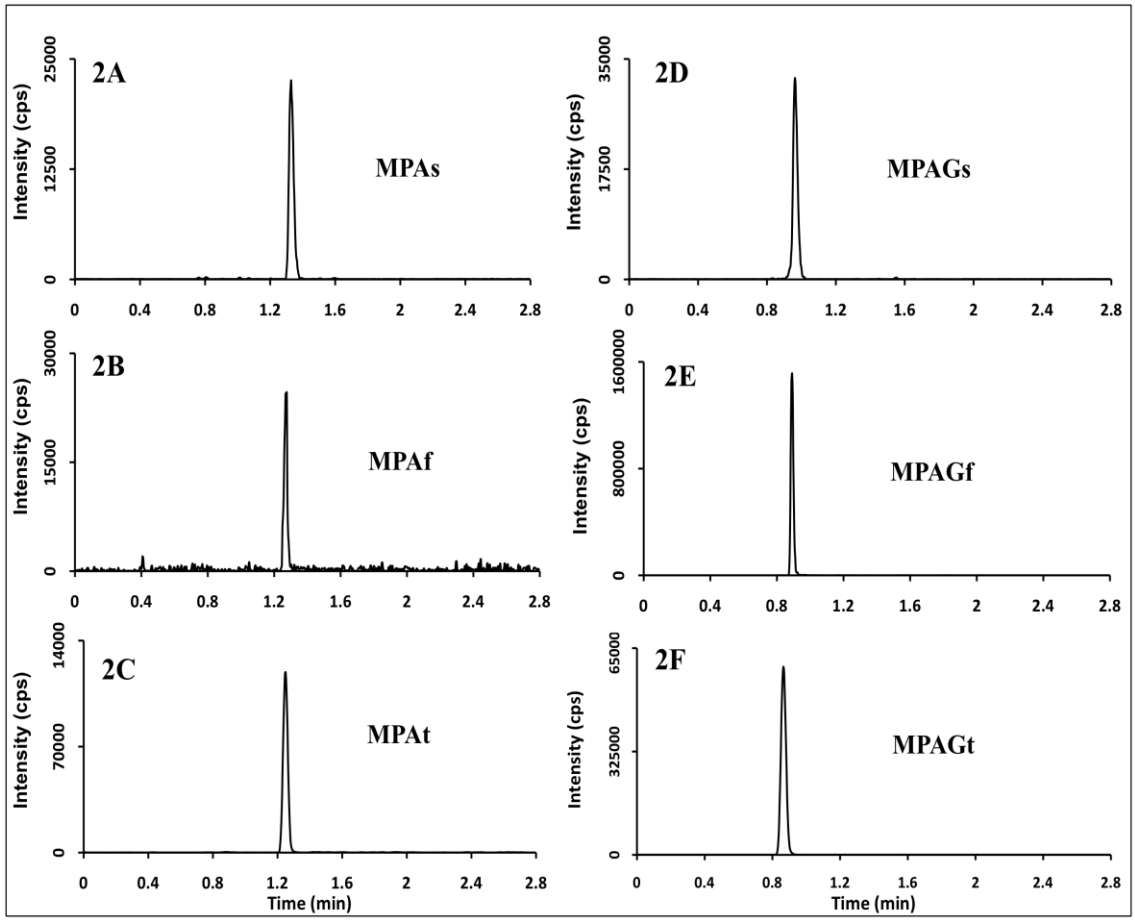
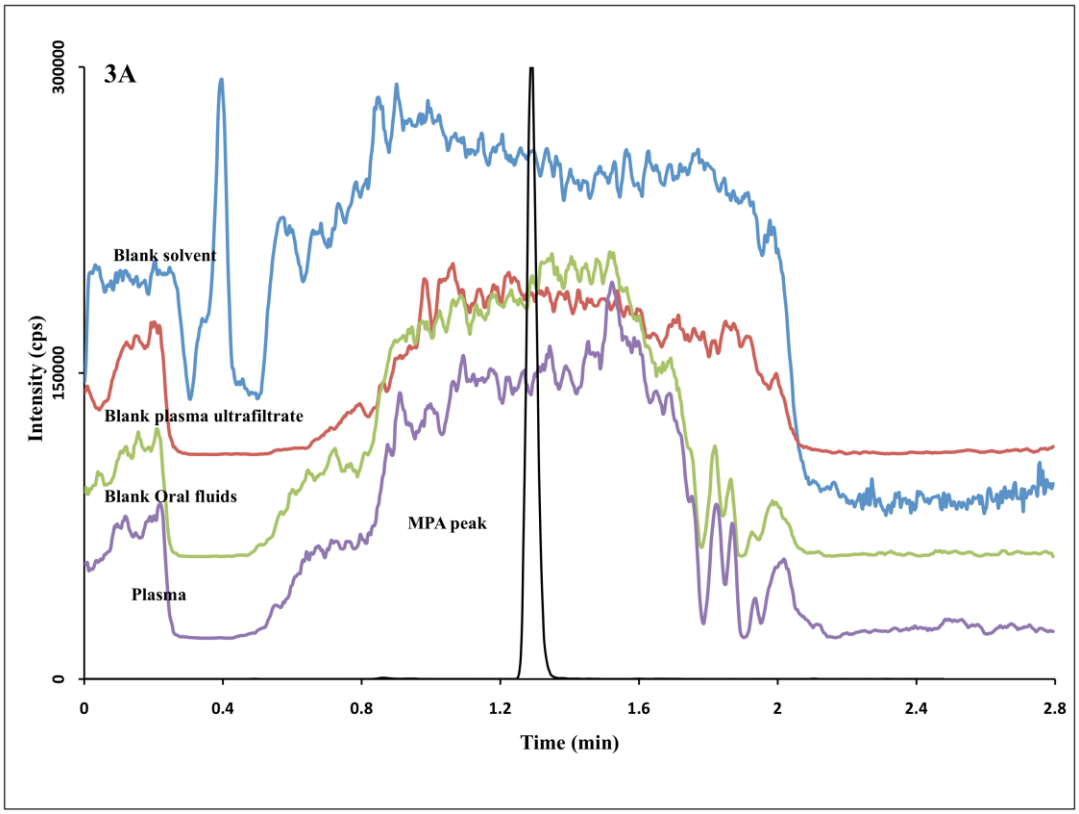


Figure 4-3: Composite chromatogram of traces obtained from continues post-column infusion chromatograms of MPA (3A), MPAG (3B) and the internal standard (3C) overlaid on a chromatograms of injections of blank injections of mobile phase, oral fluids, plasma ultra filtrate and plasma rat and human plasma. There is now area of ion suppression or enhancement is seen at elution areas of the analytes.



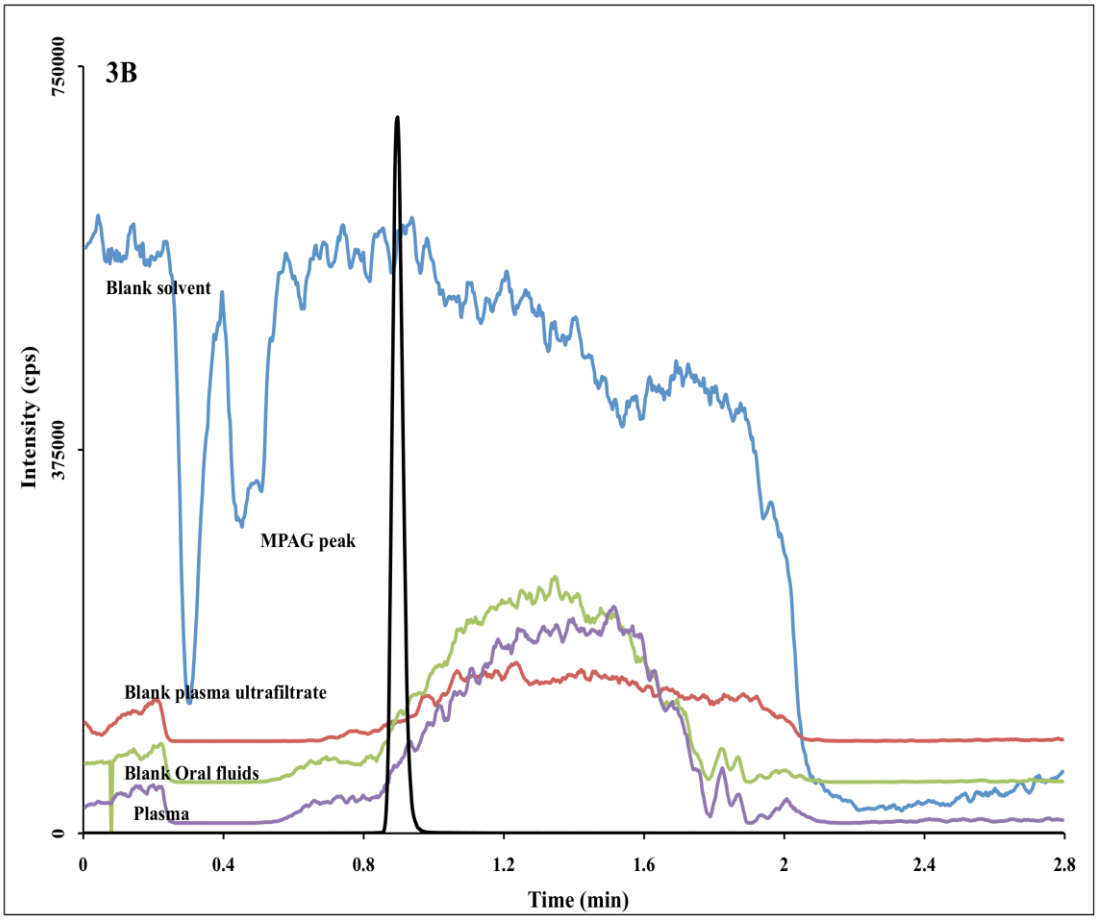
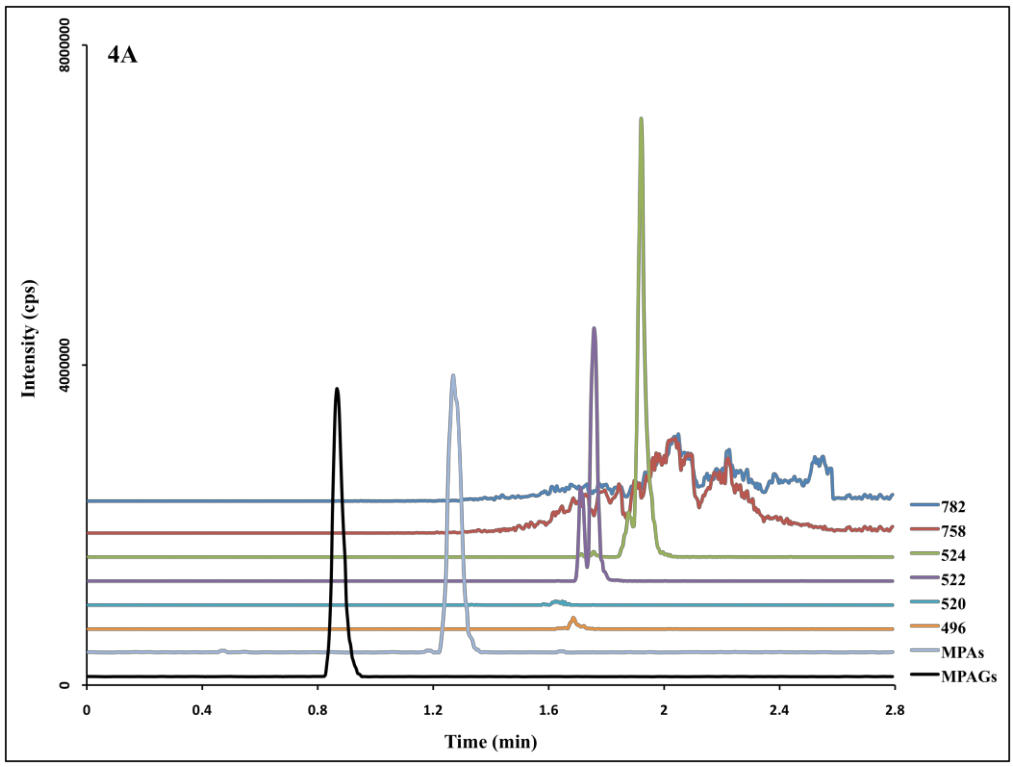
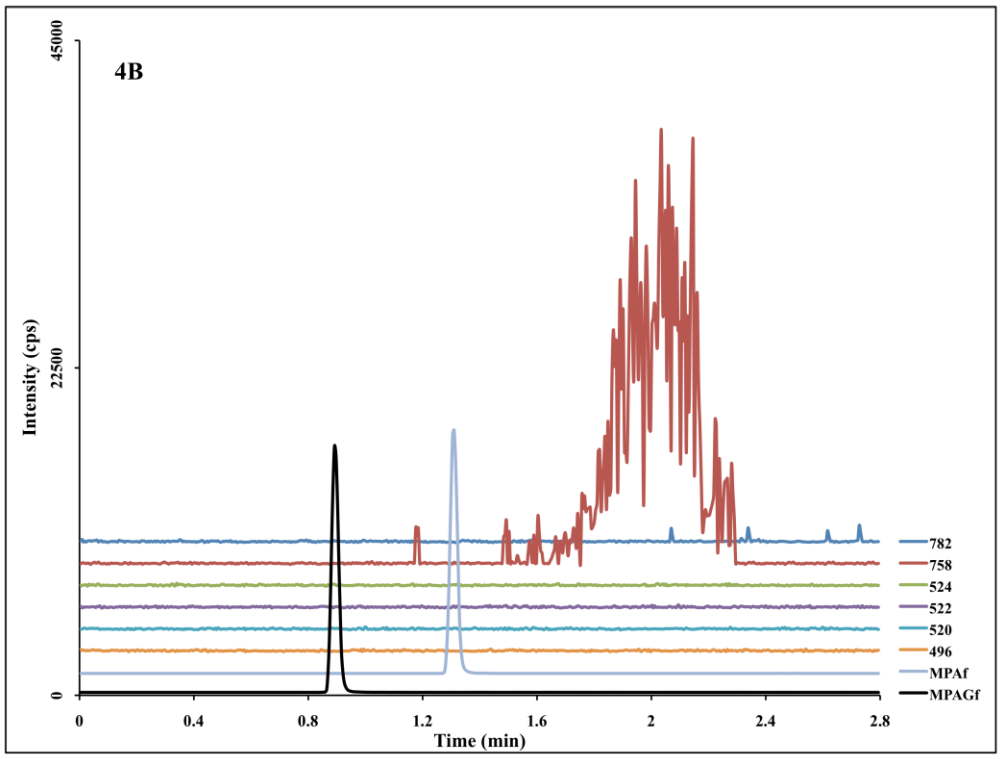

3B
750000
375000
0
Intensity (cps)
Blank solvent
MPAG peak
Blank plasma ultrafiltrate
Blank Oral fluids
Plasma
0
0.4
0.8
1.2
1.6
2
2.4
2.8
Time (min)

Figure 4-4: Chromatograms depicting traces of phospholipids obtained from injecting pooled blank samples of rat oral fluids (4A), plasma ultrafiltrate (4B) and plasma (4C). MRM transition of each individual phospholipids species is shown on the right side of the graph. Peaks of MPA and MPAG are also shown.




4B
45000
22500
0
Intensity (cps)
0
0.4
0.8
1.2
1.6
2
2.4
2.8
Time (min)
782
758
524
522
520
496
MPAf
MPAGf

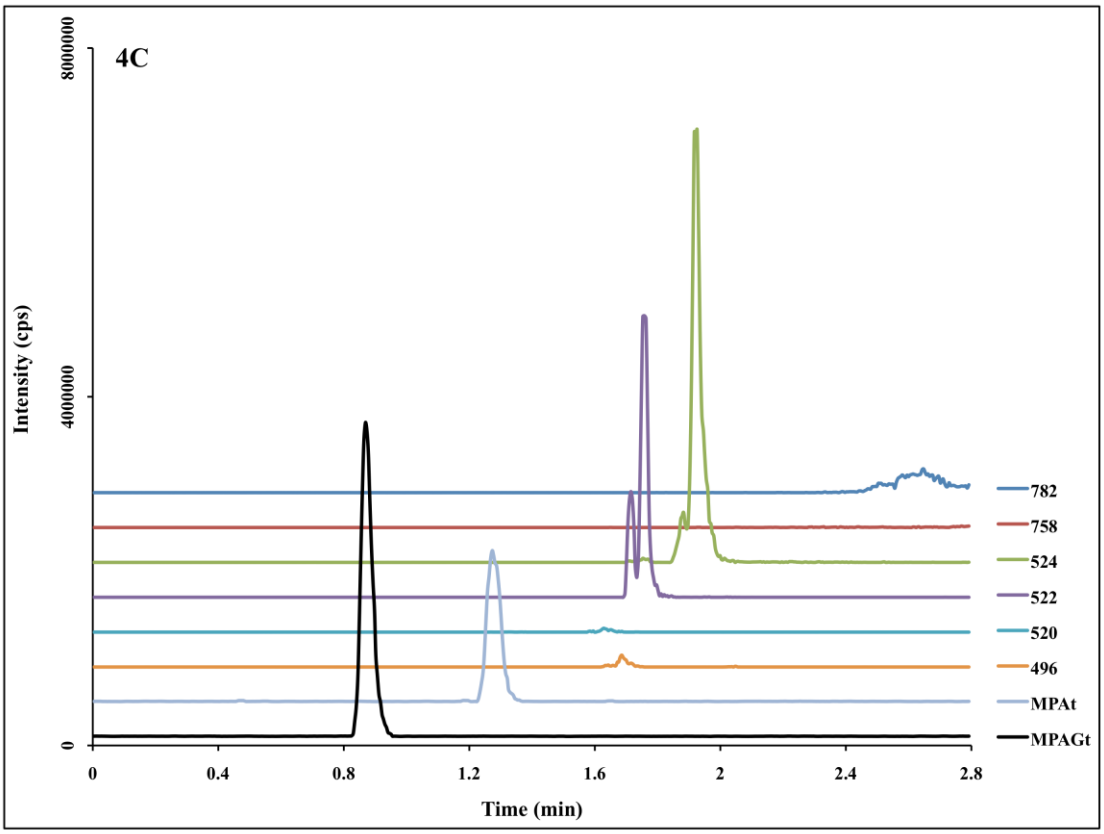
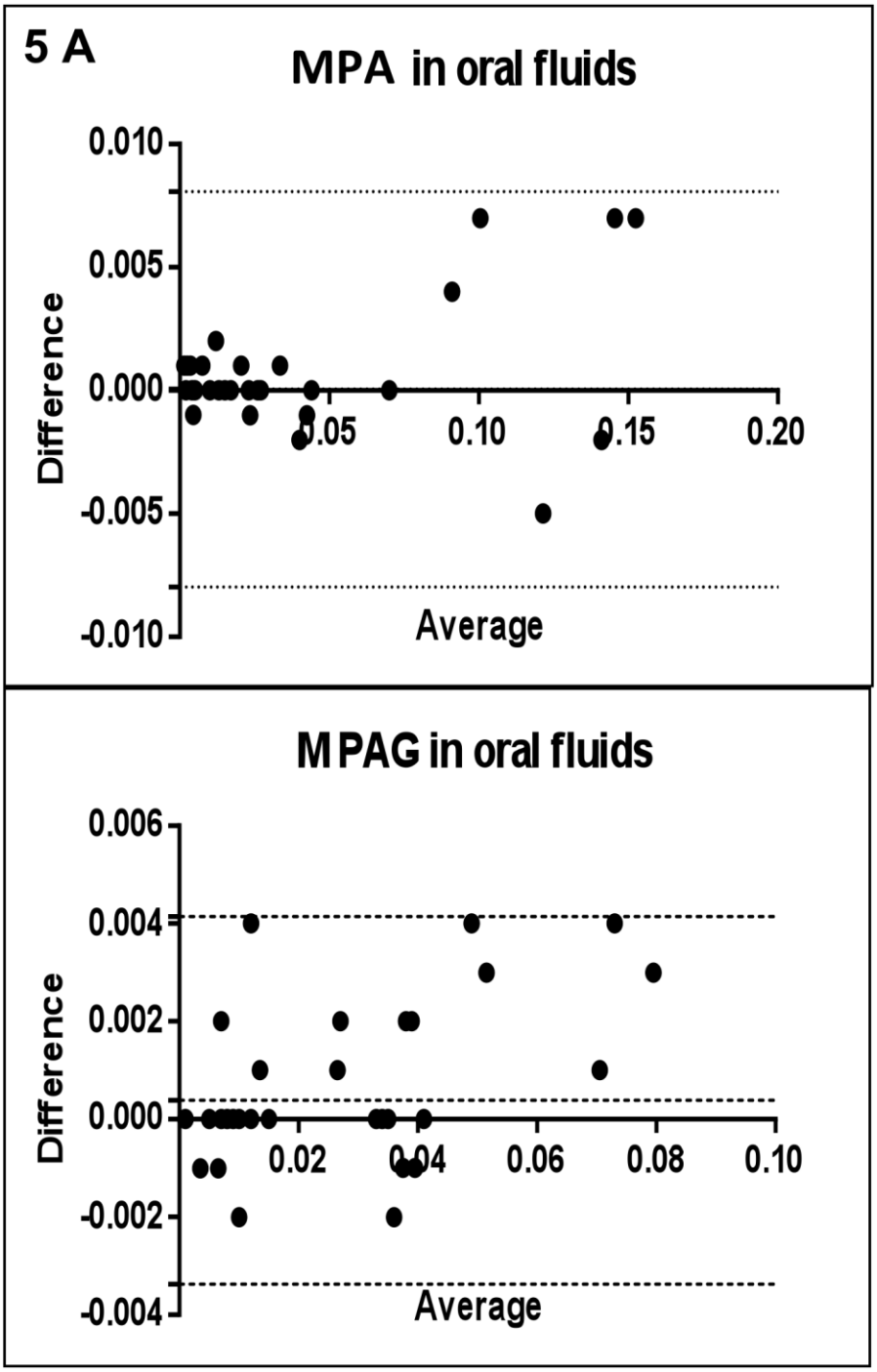
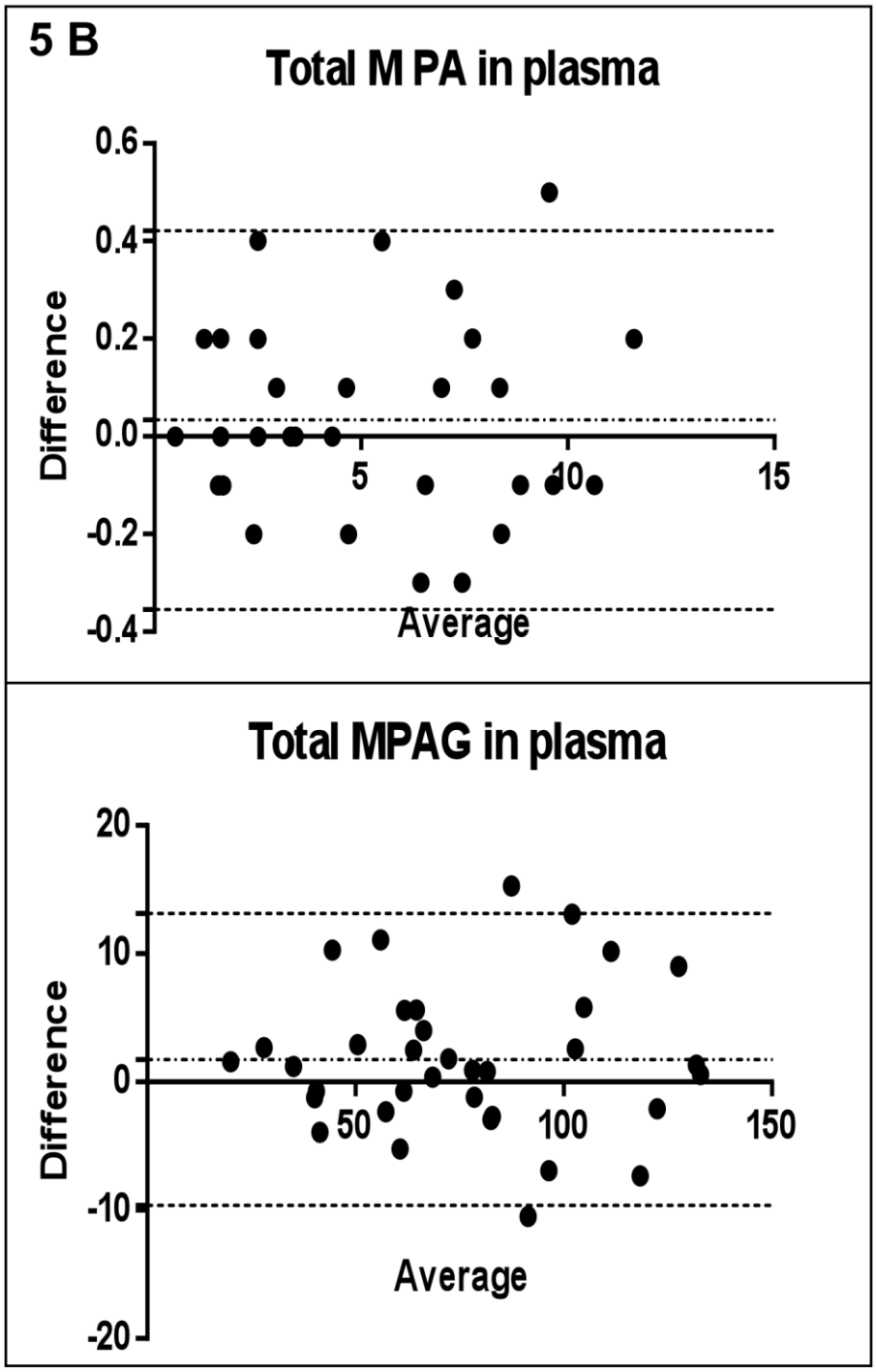
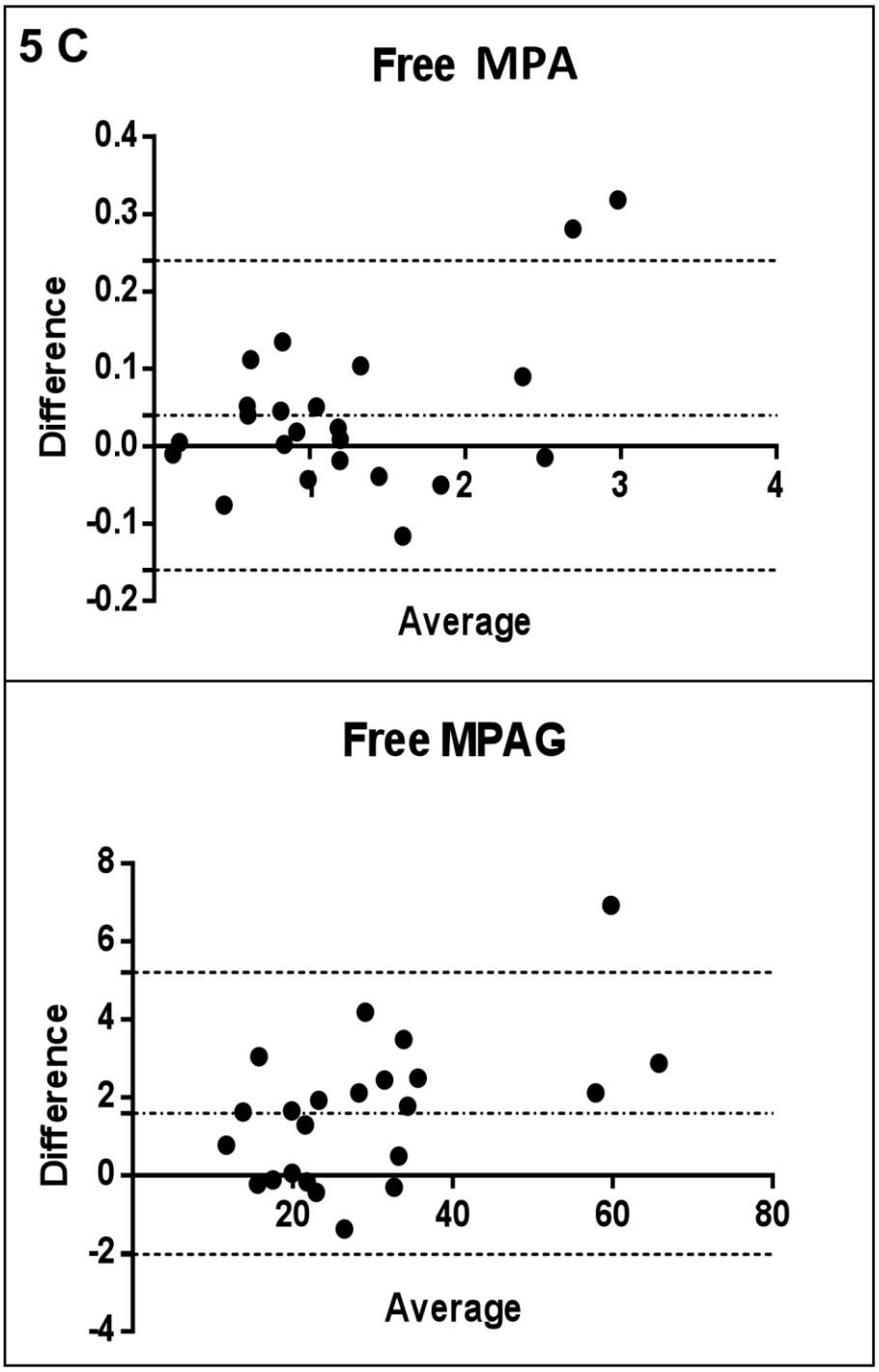

4C
8000000
4000000
0
Intensity (cps)
0
0.4
0.8
1.2
1.6
2
2.4
2.8
Time (min)
782
758
524
522
520
496
MPAt
MPAGt

Figure 5: Bland-Altman plot of difference between the repeated measurements plotted against mean differences.




5 B
Total M PA in plasma
0.6
0.4
0.2
0.0
-0.2
-0.4
Difference
5
10
15
Average
Total MPAG in plasma
20
10
0
-10
-20
Difference
50
100
150
Average


5 C
Free MPA
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
Difference
1
2
3
4
Average
Free MPAG
8
6
4
2
0
-2
-4
Difference
20
40
60
80
Average

References

1. Andreoni, K.A., et al., *Kidney and pancreas transplantation in the United States, 1996-2005*. Am J Transplant, 2007. **7**(5 Pt 2): p. 1359-75.

2. Staatz, C.E. and S.E. Tett, *Clinical pharmacokinetics and pharmacodynamics of mycophenolate in solid organ transplant recipients*. Clin Pharmacokinet, 2007. **46**(1): p. 13-58.

3. Mohammadpour, N., et al., *A review on therapeutic drug monitoring of immunosuppressant drugs*. Iran J Basic Med Sci, 2011. **14**(6): p. 485-98.

4. Wiesen, M.H., et al., *Liquid chromatography-tandem mass spectrometry method for the quantification of mycophenolic acid and its phenolic glucuronide in saliva and plasma using a standardized saliva collection device*. J Chromatogr A, 2012. **1241**: p. 52-9.

5. Shen, B., et al., *Determination of total, free and saliva mycophenolic acid with a LC-MS/MS method: application to pharmacokinetic study in healthy volunteers and renal transplant patients*. J Pharm Biomed Anal, 2009. **50**(3): p. 515-21.

6. Mendonza, A.E., R.Y. Gohh, and F. Akhlaghi, *Analysis of mycophenolic acid in saliva using liquid chromatography tandem mass spectrometry*. Ther Drug Monit, 2006. **28**(3): p. 402-6.

7. Wilhelm, A.J., et al., *Analysis of mycophenolic acid in dried blood spots using reversed phase high performance liquid chromatography*. J Chromatogr B Analyt Technol Biomed Life Sci, 2009. **877**(30): p. 3916-9.

8. Schwartz, E.B. and D.A. Granger, *Transferrin enzyme immunoassay for quantitative monitoring of blood contamination in saliva*. Clin Chem, 2004. **50**(3): p. 654-6.

9. *US Department of Health and Human Services. Food and Drug Administration (FDA). Center for Drug Evaluation and Research (CDER). Guidance for Industry.Bioanalytical Method Validation* 2001.

10. Vogeser, M., et al., *Potential lack of specificity using electrospray tandem-mass spectrometry for the analysis of mycophenolic acid in serum*. Ther Drug Monit, 2001. **23**(6): p. 722-4.

11. Zhang, G. and C.E. Wujcik, *Overcoming ionization effects through chromatography: a case study for the ESI-LC-MS/MS quantitation of a hydrophobic therapeutic agent in human serum using a stable-label internal*

standard. J Chromatogr B Analyt Technol Biomed Life Sci, 2009. **877**(22): p. 2003-10.

12. Chambers, E., et al., *Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses*. J Chromatogr B Analyt Technol Biomed Life Sci, 2007. **852**(1-2): p. 22-34.

13. Dams, R., et al., *Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: influence of ionization type, sample preparation, and biofluid*. J Am Soc Mass Spectrom, 2003. **14**(11): p. 1290-4.

14. Hardinger, K.L., et al., *Influence of pill burden and drug cost on renal function after transplantation*. Pharmacotherapy, 2012. **32**(5): p. 427-32.

15. Jacubeit, T., D. Drisch, and E. Weber, *Risk factors as reflected by an intensive drug monitoring system*. Agents Actions Suppl, 1990. **29**: p. 117-25.

16. van Amsterdam, P., et al., *The European Bioanalysis Forum community's evaluation, interpretation and implementation of the European Medicines Agency guideline on Bioanalytical Method Validation*. Bioanalysis, 2013. **5**(6): p. 645-59.

Chapter 5 M MANUSCRIPT V

To be submitted to Clinical Pharmacokinetics

Therapeutic Drug Monitoring of Mycophenolic Acid in Oral Fluid samples from Kidney Transplant Recipients

Mwlod Ghareeb[1], Reginald Y. Gohh [2] , Fatemeh Akhlaghi[1]

DEPARTMENT AND INSTITUTION

[1]Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, RI
[2] Division of Organ Transplantation, Rhode Island Hospital, Warren Alpert Medical School of Brown University, Providence, Rhode Island, USA.

Address correspondence to: Fatemeh Akhlaghi, PharmD, PhD, Clinical Pharmacokinetics Research Laboratory, Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, 495A College of Pharmacy, 7 Greenhouse Road, Kingston, RI 02881, USA. Phone: (401) 874 9205. Fax: (401) 874 5787. Email: fatemeh@uri.edu

DISCLOSURE

No conflict of interest is declared

ABBREVIATIONS

C_0: pre-dose concentration, C_2: Two post dose concentration, LC-MS/MS: Liquid chromatography tandem mass spectrometry, LLOQ: Lower limit of quantification, MPA: Mycophenolic acid, MPAf: Unbound mycophenolic acid concentration in plasma, MPAof: Oral fluid mycophenolic acid concentration, MPAt: Total mycophenolic acid concentration in plasma, MPAG: Mycophenolic acid glucuronide, MPAGf: Unbound mycophenolic acid glucuronide concentration in plasma MPAof: Oral fluid mycophenolic acid glucuronide concentration, MPAt: Total mycophenolic acid glucuronide concentration in plasma, OF: Oral fluid, TDM: Therapeutic drug monitoring, TRN: Transferrin

Abstract

Mycophenolic acid (MPA) is widely described immunosuppressive agent for solid organ transplant patients. It has a narrow therapeutic index. Therefore routine therapeutic drug monitoring (TDM) is recommended. Since the free drug fraction is responsible for pharmacological and toxic effect, quantifying unbound fraction might be more sensible. Quantifying plasma-unbound fraction is costly and labor intensive. However, drugs presented in oral fluid (OF) are considered a preventative of plasma free fraction. Therefore, oral fluid drug concentration may provide a mean for estimating unbound fraction with simple sample preparation procedures in noninvasively collected samples. In this paper, the concentration of MPA and its glucuronide metabolites (MPAG) were quantified in OF, plasma and plasma ultra-filtrate.

The correlation between MPA and MPAG concentrations in three matrices was investigated. Moreover, factors that may affect such correlation, including sampling time, salivary blood contamination, and food were investigated.

Introduction

In United States, in year 2005, about 87% of kidney and pancreas transplant patients were prescribed Mycophenolic acid (MPA) at hospital discharge [1]. MPA is a substrate for uridine diphosphate glucuronosyltransferases (UGTs). It is metabolized to a major inactive metabolite mycophenolic acid β-D-glucuronide (MPAG) and the minor but pharmacologically active metabolites mycophenolic acid acyl-β-D-glucuronide (AcMPAG) [2]. About 97-99% of MPA binds to plasma protein [2]. MPAG metabolites also bind to plasma protein and increased level MPAG, as in

patients with compromised renal function, may increases by 3-6 folds resulting displacement of MPA from plasma protein binding sites [2]. As a result, MPA free fraction may increase up to 7% [2]. Because of the narrow therapeutic index, therapeutic drug monitoring of MPA is recommended. Currently, plasma obtained through venipuncture is used for TDM of MPA [3]. Due to the invasive nature of blood sampling, alternative matrices were investigated, including dried blood spot [4] and OF [5-7].

The free fraction of a drug is responsible for pharmacological and toxicological effects [2,8,9], Therefore, measuring drug concentrations in OF may provide a better prediction of clinical outcomes and toxicity. The concentration of a drug in OF represents free drug concentration [10-12]. Thus, salivary drug level measurements are much easier and faster compared to quantifying free drug concentrations in plasma [6,7,13]. Mycophenolic acid is a small molecule with a molecular weight of 320.3, has a lipophilic nature (LogD = 2.57 and 0.75 at pH 5.5 and 7.4, respectively) [14]. These characteristics facilitate its movement through biological and entering OF [11,15]. In this paper, the association between MPA and MPAG metabolites in oral fluid, plasma, and plasma ultra-filtrate was studied.

Study population

Samples included collected in two studies from patients attending kidney transplant clinics and recruited in two studies. Patients' were on triple immunosuppressants regimen included tacrolimus or sirolimus, prednisone, and mycophenolic acid. Before conducting the studies, protocols were reviewed and approved by Institutional Review Board at Rhode Island Hospital (Providence, RI).

Patients Samples

After the physical examination by the physician, patients were asked to sign the informed consent. In the first study, patients were asked to give about 4mL venous blood samples, collected ethylenediaminetetraacetic acid (EDTA), and matching OF samples collected sporadically at certain time points, including, pre-dose (time 0 = C_0). In the second study, C_0 blood samples were collected with 3 matching OF samples collected at resting, 5 min after mouth rinsing using bottled water, and immediately after giving a saliva stimulant (commercial sour candy). Following, the patients were given vouchers for free breakfast and asked to report back at the study location shortly before 2 hours after dose (C_2) sampling time when blood a sample and correspond OF samples were collected. The OF samples were collected by passive drool into siliconized plastic cups. Blood and OF samples kept on dry ice till transferred to the Biomedical and Pharmaceutical Sciences (BPS) department at University of Rhode Island and stored at – 80 °C till analyzed.

Statistical data analysis

Statistical analysis was performed using the SPSS software (version 19.0, SPSS Inc., Chicago, IL, USA). Normal distribution of the data was checked graphically and confirmed with the Shapiro-Wilk test, and nonparametric tests were used whenever needed.

Measuring MPA and MPAG in OF and blood

Concentrations of MPA and MPAG in OF (MPAof and MPAGof, respectively), plasma (MPAt and MPAGt, respectively) and plasma ultrafiltrate (MPAf and MPAGf, respectively) were measured using a validated LC-MS/MS method (not published,

Chapter IV). In brief, an Acquity UPLC BEH C18 column (Waters Corp) was utilized as a stationary phase. Full chromatography separation between MPA and MPAG was achieved within a run time of 2.8 min using gradient elution delivered at 0.350 mL/ min flow rate. The mobile phase consisted of water containing 95:5% water: acetonitrile (ACN) mixture contained 2 mM ammonium acetate / 0.1% (v/v) formic acid (Solvent A); and ACN containing 2 mM ammonium acetate / 0.1% (v/v) formic acid (Solvent B).

Sample preparation involved extracting 50 µL of OF or plasma patients' samples, calibration curve standards, quality controls (QCs), blank, with 100µL or 200 µL of ACN precipitating solvent containing the internal standard, respectively, in 96-well plates. After shake mix and centrifugation, the supernatant then transferred to a new plate using a multichannel pipette. For MPAf and MPAGf quantification, 300uL of patients' plasma samples, calibration curve standards, QCs and blank were loaded into the ultra-filtration devices and centrifuged following manufacturer's recommendation (Centrifree® ultrafiltration device from EMD Millipore, Merck KGaA, Darmstadt, Germany). One part of ultra-filtrate was diluted with five parts of 50% ACN, briefly vortex mix and transferred to 96-wells plate. The injection volumes were 5µL for OF and plasma; and 10µL for plasma ultra-filtrate.

Salivary pH and blood contamination

Orion STAR A111 pH meter equipped with Micro Electrode from Thermo Scientific (Waltham, MA, USA) was used to measure salivary pH. To assess and quantify possible salivary blood contamination, transferrin kit from Salimetrics LLC (State College, PA, USA) was used following manufacture's recommendations [16].

Transferrin (TRN) quantification was performed using SpectraMax M5e Microplate Reader (Sunnyvale, CA, USA).

Results

Clinical studies

The demographic information of studies participants is showed in **Table 5.1.** In total 267 samples were collected. Transferrin level higher than the recommended limit (>1mg/dL), was seen in 81 OF samples, therefore, excluded from further analysis. In the first study, intensive sampling was used to obtain blood and OF samples at rest with a total of 144 samples included in the statistical analysis. In the second study, blood and OF samples were collected at C_0 and C_2, a total of 142 samples were included. All included samples had MPA concentrations higher than the lower limit of quantification (LLOQ) Twenty-two samples had MPAG concentration lower LLOQ, but higher than the lower limit of detection (LLOD).

Results and discussion

12 hours profile study

Summary statistics of MPA and MPAG concentrations in all matrices is showed in **Table 5.2**. As can be seen in **Table 5.2, the** plasma concentration of MPAG is about 13 times higher than corresponding MPA (mean± STD, 46.03±45.50 vs. 3.43±2.50, respectively) which has been previously reported [2]. A comparable percentage of MPA unbound fraction (MPAf, mean± STD, 6.99±6.14) and MPAGf (mean± STD, 7.03±4.79) was observed. Conversely, a smaller percentage of MPAG (0.07±0.06) detected in OF compared to MPA (0.78±0.55). In fact, the oral fluid concentration of

MPA was about 10 folds higher than MPAG. The lower salivary concentration of MPAGs can be attributed to lower lipophilicity and higher molecular weight. High variability in MPA and MPAG is obvious in all matrices which have is previously reported [17].

The area under the plasma concentration-time curve (AUC) and the maximum concentration are the best parameters to measure to estimate exposure to predict clinical outcome and toxicity [18]. Nevertheless, estimating AUC and C_{max} requires multiple sampling over a dosing interval period of up to 12 hrs, which is impractical for routine TDM using venipuncture blood sampling. Owing to the ease of sample collection, and possible self-home sampling [19]; and significantly reduced sample cost [19,20], oral fluid as a medium has the potential to make calculating AUC feasible. In this study, the AUC_{0-12} for OF, unbound and total MPA and MPAG were calculated. Summary statistics of AUC_{0-12} is presented in **Table 5.3**.

Plots of mean concentrations versus time of MPA and MPAG in three matrices are shown in **Figure 5.1A** and **5.1B**, respectively**.** The mean (t_{max}) of MPA was at around one-hour after dose. A second peak is seen around four-hours after dose representing enterohepatic recirculation of MPAG back to MPA [21].

Good correlation can be seen when individual's AUC_{0-12} of MPA in OF samples plotted against unbound and total MPA, (**Figure 2.1A and 2.2A,** respectively). Weaker association is seen between MPAf and MPAt (**Figure 2.3A**). In contrast, only unbound and total MPAG concentrations showed reasonable association (**Figure 2.3B**).

Representative diagrams of the mean TRN and pH concentrations are shown in **Figure 5.3**. The mean TRN concentration had an elevated level in pre-dose and started to decline and level out after one hour after the dose. In the other hand, pH level showed a random pattern.

2 hours profile study

In the second study, the aim was to investigate the effect of different sampling conditions on the quality of OF samples obtained before (C_0) and two hours (C_2) after taking morning medications. The samples were collected either at rest, after mouth rinsing and after giving OF stimulants. In addition, the effect of salivary blood contamination on quality and amount of the MPA and MPAG was studied. Shapiro-Wilk test revealed the abnormal distribution of salivary pH; TRN, MPA, and MPAG levels. Therefore, nonparametric tests were used.

Effect of blood salivary contamination on endogenous substances has been studied [22]. According to the authors, high TRN level was associated with higher dehydroepiandrosterone but had a mitigated effect on the salivary level of compounds studied, cortisol and testosterone. For MPA, high concentrations in OF samples collected at C_0 combined with elevated TRN level have been reported [7]. Similar finding is seen in this study, where significantly higher TRN levels in C_0 resting and rinsed samples (**Figures 5.4.A and 5.4.B, respectively.)** compared with C_2 resting and rinsed samples. No significant difference between stimulated OF samples collected at both time points (**Figure 5.4.C**).

In addition, significant differences in TRN concentration in resting and rinsed samples compared with stimulated samples only seen in C_0 (**Figure 5.5.C**). However, the concentration of MPA was not significantly different between resting, rinsed, and stimulated OF samples (**Figure 5.5.A)**, which may suggest limited/ no effect of TRN level on MPA salivary concentration. No difference in TRN level is seen in OF samples collected at C_2 (**Figure 5.5.D**). This may indicate the abundance of TRN in fasting samples, that even mouth rising was not enough to reduce salivary blood contamination.

Conclusion

In samples obtained from stable renal transplant recipients good correlation between $AUC0\text{-}_{12}$ of MPA in OF samples and unbound and total MPA. In contrast, a weak association between MPAG concentrations in oral fluids with total and unbound plasma fraction. Limited effect of TRN level in OF on MPA concentration.

Table 5-1: Demographic information of study population

Gender	**Female = 35**	**Male = 24**	
	Mean ± STD	**Min.**	**Max.**
Age	50 ± 14	21	73
Patient's weight (Kg)	92.34 ± 18.74	52.2	148.3
Creatinin clearance	76 ± 25	31	167
cholesterol (mg/dl)	185 ± 42	115	268
Creatinin clearance	76 ± 25	31	167
Protein total (mg/dl)	6.8 ± 0.4	5.7	7.7
Blood creatinine level (mg/dl)	1.32 ± 0.41	0.66	2.54
ALT (iu/L)	25 ± 67	15	8
AST (iu/L)	31 ± 333	53	9
bilirubin (mg/dl)	0.4 ± 1.3	0.2	0.2
Blood glucose level (mg/dl)	127 ± 419	55	54

Table 5-2: statistic summary of measured parameters

		N	Mean	STD	Median	Min.	Max.
Oral fluids	pH	144	7.57	7.69	0.55	6.050	8.49
	Transferrin concentration (mg/dL)	144	0.31	0.27	0.22	0.060	0.98
	MPA conc (mg/L)	144	0.02	0.01	0.03	0.001	0.14
	MPAG conc (mg/L)	144	0.03	0.03	0.02	0.004	0.13
Plasma ultrafiltrate	MPA conc (mg/L)	144	0.21	0.15	0.23	0.013	1.31
	MPAG conc (mg/L)	144	3.27	2.7	2.12	0.508	12.83
Plasma	MPA conc (mg/L)	144	3.43	2.5	2.76	0.2	14.6
	MPAG conc (mg/L)	144	46.03	45.5	16.07	10.5	93.6
% Oral fluid fraction	MPA	144	0.78	0.55	0.70	0.030	3.71
	MPAG	144	0.07	0.06	0.06	0.007	0.35
% Free Fraction	MPA	144	7.03	4.79	6.86	1.368	50.59
	MPAG	144	6.99	6.14	3.22	3.279	25.56

Table 5-3: Shows statistics of $AUC_{0\text{-}12}$ of MPA and MPAG in oral fluids, unbound fraction and total concentration in plasma.

	$AUC_{0\text{-}12}$ (mg × h × L^{-1})					
	MPA			MPAG		
	Oral fluids	Ultrafiltrate	Plasma	Oral fluids	Ultrafiltrate	Plasma
Mean	0.53	1.40	22.22	0.61	19.08	257.92
STD	0.46	1.44	14.62	0.42	15.29	110.69
Median	0.38	0.97	15.82	0.49	13.12	237.56

Figure 5-1: Salivary, unbound and total concentration (mg/L) of mycophenolic acid (4.1A) and glucuronide metabolites (4.1B) versus time; data are expressed as mean and error bars represent standard error.

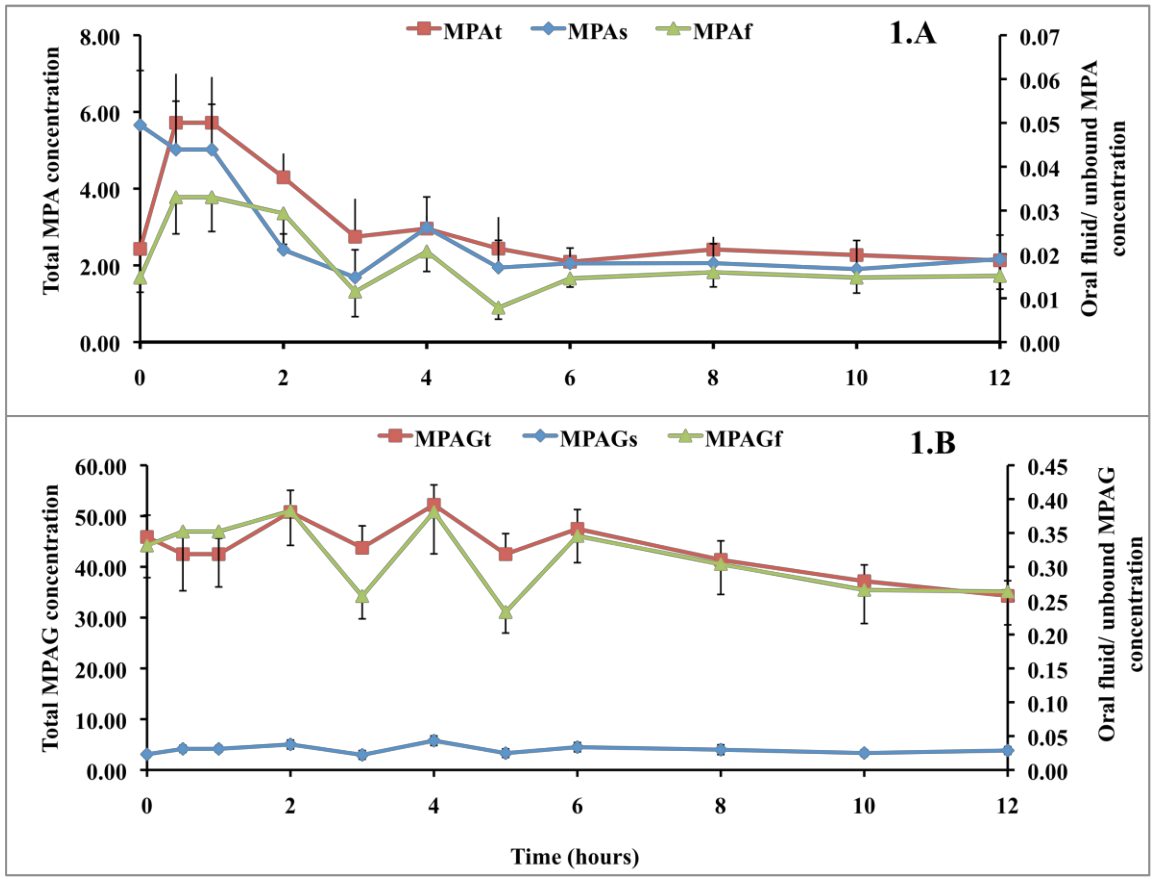


Figure 5-2 Plots of mean AUC_{0-12} of mycophenolic acid (4.2.A-1, 4.2.A-2, and 4.2.A-3) and glucuronide metabolites (42.B-1, 4.2.B-2, and 4.2.B-3,) in oral fluids vs. unbound fraction; in oral fluids vs. total concentration ; and total vs. unbound fraction, respectively.

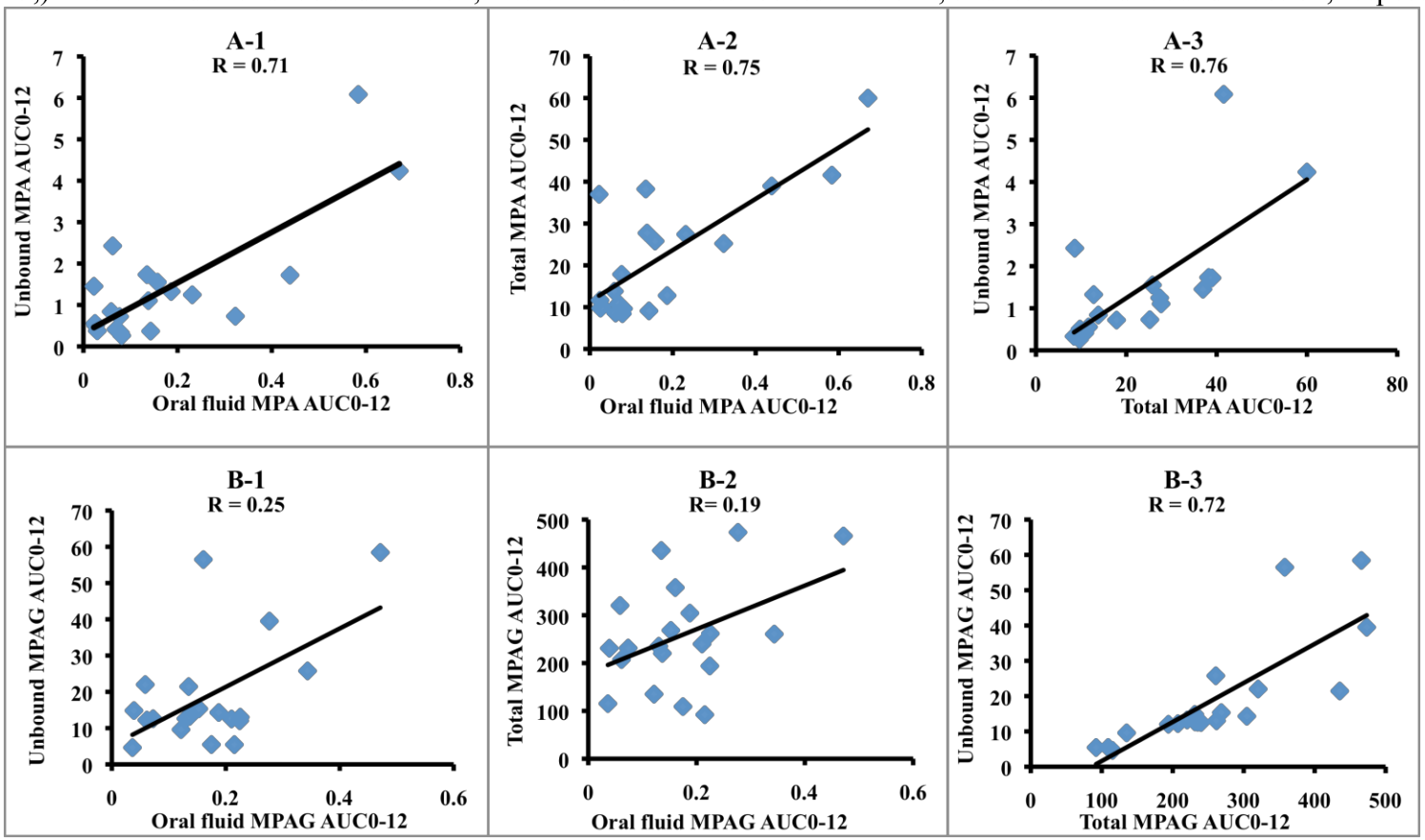


Figure 5-3: Salivary transferrin concentration (4.3.A) and pH levels (4.3.B) vs. time profiles; data are expressed as mean. The error bars represent standard error.

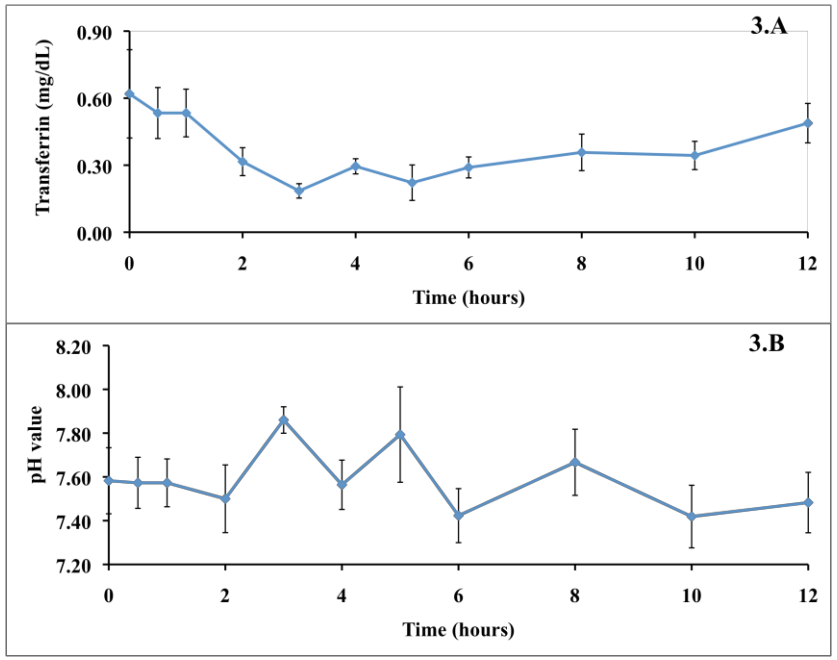


Figure 5-4. Box plots compare the transferrin concentration at pre and two hours after dose and with different sampling conditions at resting (4.A), rinsed (4.B), and stimulated (4.C)

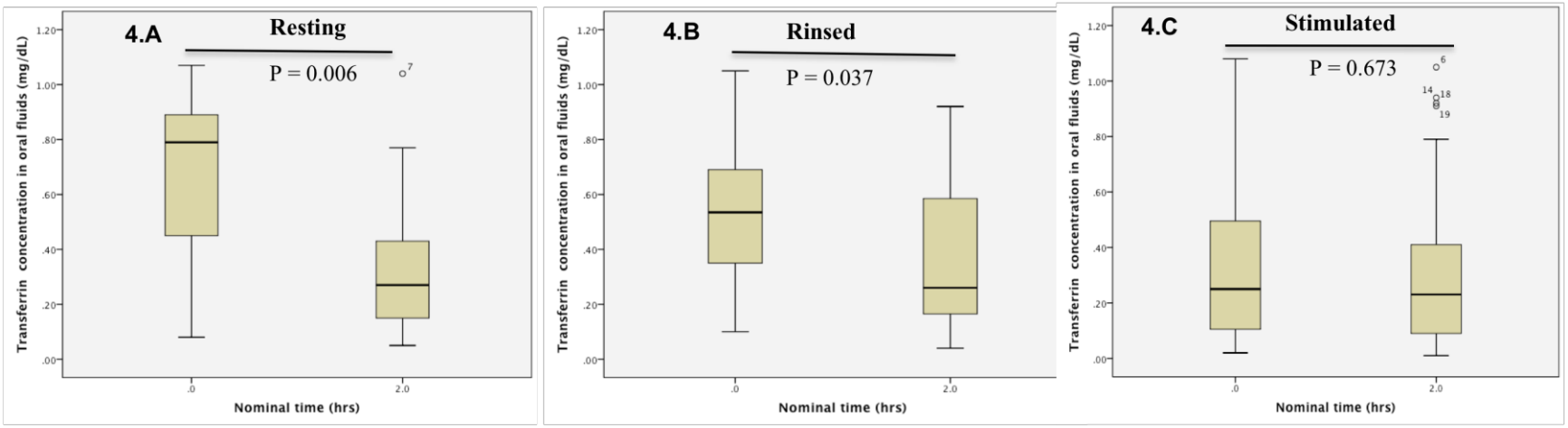
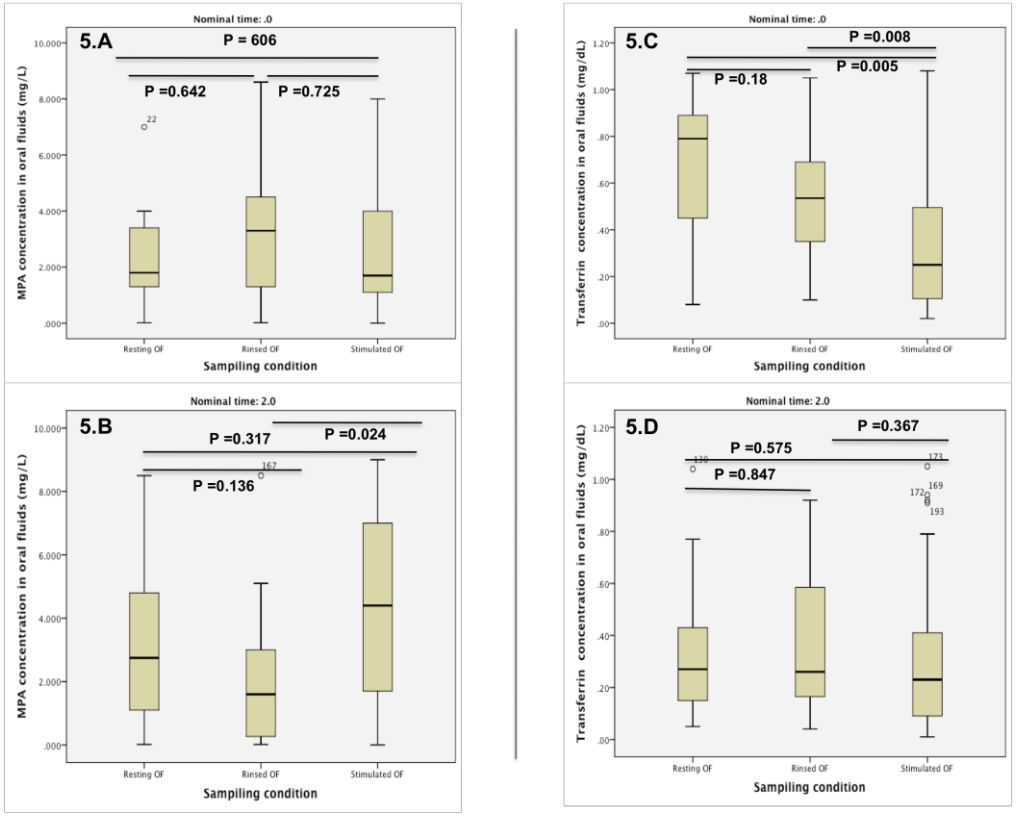


Figure 5-5. Box plots at left column show mycophenolic acid concentrations in resting, rinsed, and stimulated oral fluid samples at pre-dose (5.A) and two-hours after dose (5.B). In the right column, the box plots show transferrin level at pre-dose (5.C) and two-hours after dose (5.D)



References

1. Andreoni KA, Brayman KL, Guidinger MK, Sommers CM, Sung RS. Kidney and pancreas transplantation in the United States, 1996-2005. *Am. J. Transplant.*, 7(5 Pt 2), 1359-1375 (2007).

2. Staatz CE, Tett SE. Clinical pharmacokinetics and pharmacodynamics of mycophenolate in solid organ transplant recipients. *Clinical pharmacokinetics*, 46(1), 13-58 (2007).

3. Mohammadpour N, Elyasi S, Vahdati N, Mohammadpour AH, Shamsara J. A review on therapeutic drug monitoring of immunosuppressant drugs. *Iranian journal of basic medical sciences*, 14(6), 485-498 (2011).

4. Wilhelm AJ, den Burger JC, Chahbouni A, Vos RM, Sinjewel A. Analysis of mycophenolic acid in dried blood spots using reversed phase high performance liquid chromatography. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 877(30), 3916-3919 (2009).

5. Wiesen MH, Farowski F, Feldkotter M, Hoppe B, Muller C. Liquid chromatography-tandem mass spectrometry method for the quantification of mycophenolic acid and its phenolic glucuronide in saliva and plasma using a standardized saliva collection device. *Journal of chromatography. A*, 1241, 52-59 (2012).

6. Shen B, Li S, Zhang Y *et al*. Determination of total, free and saliva mycophenolic acid with a LC-MS/MS method: application to pharmacokinetic study in healthy volunteers and renal transplant patients. *Journal of pharmaceutical and biomedical analysis*, 50(3), 515-521 (2009).

7. Mendonza AE, Gohh RY, Akhlaghi F. Analysis of mycophenolic acid in saliva using liquid chromatography tandem mass spectrometry. *Therapeutic drug monitoring*, 28(3), 402-406 (2006).

8. Zahir H, McCaughan G, Gleeson M, Nand RA, McLachlan AJ. Changes in tacrolimus distribution in blood and plasma protein binding following liver transplantation. *Therapeutic drug monitoring*, 26(5), 506-515 (2004).

9. Akhlaghi F, Ashley J, Keogh A, Brown K. Cyclosporine plasma unbound fraction in heart and lung transplantation recipients. *Therapeutic drug monitoring*, 21(1), 8-16 (1999).

10. Haeckel R. Factors influencing the saliva/plasma ratio of drugs. *Ann. N. Y. Acad. Sci.*, 694, 128-142 (1993).

11. Jusko WJ, Milsap RL. Pharmacokinetic principles of drug distribution in saliva. *Ann. N. Y. Acad. Sci.*, 694, 36-47 (1993).

12. Haeckel R, Hanecke P. Application of saliva for drug monitoring. An in vivo model for transmembrane transport. *European journal of clinical chemistry and clinical biochemistry : journal of the Forum of European Clinical Chemistry Societies*, 34(3), 171-191 (1996).

13. Ionita IA, Akhlaghi F. Quantification of unbound prednisolone, prednisone, cortisol and cortisone in human plasma by ultrafiltration and direct injection into liquid chromatrography tandem mass spectrometry. *Annals of clinical biochemistry*, 47(Pt 4), 350-357 (2010).

14. ChemSpider / Search and share chemistry. (Royal Society of Chemistry) http://www.chemspider.com

15. Feller K, le Petit G. On the distribution of drugs in saliva and blood plasma. *International journal of clinical pharmacology and biopharmacy*, 15(10), 468-469 (1977).

16. Schwartz EB, Granger DA. Transferrin enzyme immunoassay for quantitative monitoring of blood contamination in saliva. *Clinical chemistry*, 50(3), 654-656 (2004).

17. Bergmann TK, Barraclough KA, Lee KJ, Staatz CE. Clinical pharmacokinetics and pharmacodynamics of prednisolone and prednisone in solid organ transplantation. *Clinical pharmacokinetics*, 51(11), 711-741 (2012).

18. Schiff J, Cole E, Cantarovich M. Therapeutic monitoring of calcineurin inhibitors for the nephrologist. *Clinical journal of the American Society of Nephrology : CJASN*, 2(2), 374-384 (2007).

19. Tennison M, Ali I, Miles MV, D'Cruz O, Vaughn B, Greenwood R. Feasibility and acceptance of salivary monitoring of antiepileptic drugs via the US Postal Service. *Therapeutic drug monitoring*, 26(3), 295-299 (2004).

20. Gorodischer R, Koren G. Salivary excretion of drugs in children: theoretical and practical issues in therapeutic drug monitoring. *Developmental pharmacology and therapeutics*, 19(4), 161-177 (1992).

21. Bullingham RE, Nicholls AJ, Kamm BR. Clinical pharmacokinetics of mycophenolate mofetil. *Clinical pharmacokinetics*, 34(6), 429-455 (1998).

22. Granger DA, Cicchetti D, Rogosch FA, Hibel LC, Teisl M, Flores E. Blood contamination in children's saliva: prevalence, stability, and impact on the measurement of salivary cortisol, testosterone, and dehydroepiandrosterone. *Psychoneuroendocrinology*, 32(6), 724-733 (2007).

Chapter 6 : MANUSCRIPT VI

Submitted to Analytical and Bioanalytical Chemistry

Development and Validation of an UPLC-MS/MS Assay for Quantitative Analysis of the Ghrelin Receptor Inverse Agonist PF-5190457 in Human or Rat Plasma and Rat Brain

Mwlod Ghareeb[1], Lorenzo Leggio[2,3], Ayman El-Kattan [4], Fatemeh Akhlaghi[1]

DEPARTMENT AND INSTITUTION

[1]Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, RI, USA, 02881
[2]Section on Clinical Psychoneuroendocrinology and Neuropsychopharmacology, Laboratory of Clinical and Translational Studies, National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD, USA; and Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Baltimore, MD, USA.
[3]Center for Alcohol and Addiction Studies, Department of Behavioral and Social Sciences, Brown University, Providence, RI, USA.
[4] Pfizer Inc., 610 Main St, Cambridge, MA, USA

Address correspondence to: Fatemeh Akhlaghi, PharmD, PhD; Clinical Pharmacokinetics Research Laboratory; Department of Biomedical and Pharmaceutical Sciences; University of Rhode Island; 495A College of Pharmacy; 7 Greenhouse Road; Kingston; RI 02881, USA. Phone: (401) 874 9205. Fax: (401) 874 5787. Email: fatemeh@uri.edu

Funding:

This work was supported by grant number 1UH2TR000963 (PIs: Akhlaghi and Leggio) from the National Center for Advancing Translational Sciences (NCATS), National Institutes of Health (NIH); and by NIH intramural funding ZIA-AA000218 (PI: Leggio) jointly supported by the Division of Intramural Clinical and Biological Research of the National Institute on Alcohol Abuse and Alcoholism (NIAAA) and the Intramural Research Program of the National Institute on Drug Abuse (NIDA).

Acknowledgment: Authors would like to thank Professor Deyu Li for his help in elucidating the fragmentation pattern of PF-5190457.

KEY WORDS

Alcoholism, bioanalytical methods, ghrelin, LC-MS/MS, PF-5190457, pharmacokinetics

ABBREVIATIONS

ACN: acetonitrile, CV: coefficient of variation IS: internal standard, LLOQ: lower limit of quantification, ME: matrix effect, MeOH: methanol, MRM: multiple reaction monitoring, MS: mass spectrometry, Mw: molecular weight, PLs: phospholipids, QCs: Quality controls, UPLC-MS/MS: ultra performance liquid chromatography tandem mass spectrometry

ABSTRACT

PF-5190457 is a ghrelin receptor inverse agonist that is currently undergoing clinical development for the treatment of alcoholism. Our aim was to develop and validate a simple and sensitive assay for quantitative analysis of PF-5190457 in human or rat plasma and rat brain using liquid chromatography-tandem mass spectrometry. The analyte and stable isotope internal standard were extracted from 50 µL plasma or rat brain homogenate by protein precipitation using 0.1% formic acid in acetonitrile. Chromatography was carried on an Acquity UPLC BEH C18 (2.1 mm X 50 mm) with 1.7 µm particle size and 130Å pore size. Flow rate was 0.5 mL/min and total chromatographic run time was 2.2 minutes. Mobile phase consisted of gradient mixture of water: acetonitrile 95:5% (v/v) containing 0.1% formic acid (Solvent A), and 100% acetonitrile containing 0.1% formic acid (Solvent B). Multiple reaction monitoring was carried out in positive electro-spray ionization mode using m/z 513.35 → 209.30 for PF-5190457 and m/z 518.47 → 214.43 for the internal standard. The recovery ranged from 102-118% with CV less than 6% for all matrices. The calibration curves for all matrices were linear over the studied concentration range ($R^2 \geq 0.998$, $n = 3$). Lower limit of quantification was 1 ng/mL in rat or human plasma and 0.75 ng/g in rat brain. Intra- and inter-run mean percent accuracy were between 85–115% and percent imprecision was $\leq 15\%$. The assays were successfully utilized to measure the concentration of PF-5190457 in pre-clinical and clinical pharmacology studies of the compound.

Introduction

Ghrelin is a 28-amino acid peptide primarily produced by the endocrine X/A-like cells of the fundus mucosa of the stomach and acts as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R1a). GHS-R1a is a G-protein coupled receptor that induces growth hormone (GH) release from the pituitary [1]. Ghrelin activates hypothalamic orexigenic neurons and inhibits anorectic neurons to induce hunger [2,3]. In humans, intravenous (IV) acetylated ghrelin administration increases appetite and food intake [4,5]. Moreover, ghrelin infusion can suppress glucose-dependent insulin secretion in rodents and humans resulting in insulin resistance [2,6]. Therefore, it is conceivable to believe that pharmacological modulation of ghrelin may be beneficial in regulating appetite and body weight or in treating type 2 diabetes mellitus.

Consistent with converging evidence illustrating that alcohol and food-seeking behaviors share common neural pathways [7,8], ghrelin signaling has been proposed as a potential novel pharmacological target for the treatment of alcoholism [9]. In mice, central ghrelin administration to reward nodes of the brain increased alcohol intake while central or peripheral administration of ghrelin receptor antagonists suppressed alcohol intake [10]. Furthermore, clinical studies from our team have shown that plasma concentrations of ghrelin were different in abstinent compared to active drinking alcohol-dependent individuals and correlated with alcohol craving [11]. Additionally, in a human laboratory setting, intravenous administration of 3 μg/kg ghrelin to alcohol-dependent, heavy-drinking individuals resulted in a significant acute increase in cue-induced alcohol craving [12]. Furthermore, there was

a positive significant correlation between post-infusion blood ghrelin levels and increased alcohol craving [12].

Generally, it appears that GHS-1Ra antagonism can possibly increase satiety and does not only result in weight loss and improvement in glycemic control but it may also be helpful for treating alcoholism. PF-5190457 is a sensitive and specific ghrelin receptor inverse agonist that is orally bioavailable [13]. It is a member of a spiro-azetidino-piperidine series that was identified through high-throughput screening by Pfizer Pharmaceuticals. PF-5190457 (Mw=512), has a measured logD value of 1.5 at pH 7.4 and a topological polar surface area of 95. Pharmacokinetics studies in rats have shown a high volume of distribution and clearance and an almost 100% fraction absorption in portal vein cannulated rats [10]. Here, we report the development and validation of a sensitive, specific and robust assay for measurements of PF-5190457 in either human or rat plasma or in rat brain homogenate using an ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) technique.

Chemicals and reagents

PF-5190457 and the internal standard (IS) PF-06340740 (stable labeled isotope) were kindly donated by Pfizer. Optima^{TM} LC/MS grade of acetonitrile, ammonium acetate, formic acid, and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was obtained using a Milli-Q Synthesis system fitted with a Q-Gard 2 Purification Pack (Millipore, Bedford, MA, USA). Drug-free K_2EDTA rat plasma or brain specimens were from Wistar rats (n=6) aged between 2 to 4 months and weighing between 300-500 grams (Bioreclamation IVT Inc., Westbury, NY, USA). Similarly, K_2EDTA human plasma from 6 subjects (3 male, 3 female) were

obtained from Bioreclamation IVT Inc.

Instruments

Eppendorf 5810 refrigerated centrifuge from Micro and Nanotechnology (Urbana, IL, USA) was used to obtain supernatants. Acquity UPLC from Waters Corp (Milford, MA, USA) connected to Xevo TQ MS mass spectrometry (Waters Corp) was used to quantify PF-5190457 concentrations. Acquity UPLC system had a binary pump and was equipped with a built-in column heater. A 20µL sample loop was used to deliver samples in partial loop injection mode. The system was controlled with MassLynx™ software (V 4.1) and data was processed using TargetLynx™ tool.

Chromatographic conditions

Chromatographic separation was carried out in an Acquity UPLC BEH C18 (2.1 mm X 50 mm) with 1.7 µm particle size and 130Å pore size analytical column (Waters Corp, Milford, MA). An Acquity UPLC BEH C18 pre-column was used to preserve the performance of the analytical column. The column was maintained at 55 °C and an auto-sampler temperature was kept at 20°C. A gradient elution method was utilized with a mobile phase consisting of water: acetonitrile 95:5% (v/v) containing 0.1% formic acid (Solvent A), and 100% acetonitrile containing 0.1% formic acid (Solvent B). The mobile phase was delivered at 0.5 mL /min flow rate. Each chromatographic cycle started and maintained at 2% solvent (B) for 0.3 min and increased gradually to 98% over 0.7 min and maintained at this level until 1.8 min. To re-equilibrate the column for the next run, the proportion of solvent (B) was decreased within 0.1 min to 2% and kept constant until the end of the run at 2.2 min. To minimize detector contamination, a diversion valve was set to deliver the first 0.60 min and from 1.10

min until the end of each run to waste. The elution time for both analyte and IS was 0.83 min.

Mass spectrometry conditions

Multiple reaction monitoring (MRM) in positive electro-spray ionization (ESI) mode was used for detection and quantification of analytes and IS. The MS scan of infused PF-5190457 detected protonated molecules [M+H]+ (m/z= 513.61) with highest intensity, followed by sodium adduct (m/z= 535.61) [M+NA]+ as seen in **Figure 1**. Therefore, the protonated form was selected. Protonated precursors were fragmented into two compounds of similar intensity with m/z values of 127.21, and 209.30; a third compound (m/z =335.35) was fragmentized with 50% intensity compared to the first two fragments (**Figure 2**). The two fragments with the highest m/z values were selected. MRM transitions were monitored (m/z, Q1> Q3); m/z, 513.35 > 209.30 transition was used for quantification while m/z, 513.35 > 335.35 transition was used as backup. M/z, 518.47 > 214.43 transition was selected for the internal standard. The proposed fragment formation of PF-5190457 is illustrated in **Figure 3**. All chemical structures were produced using ChemDraw version 14.0.0.117 from PerkinElmer Inc (Waltham, Massachusetts, USA). A gradient elution method was utilized with a mobile phase consisting of water: acetonitrile 95:5% (v/v) containing 0.1% formic acid (Solvent A), and 100% acetonitrile containing 0.1% formic acid (Solvent B).

After automatically obtaining initial mass spectrometry parameters with IntelliStart tool, manual tuning of final parameters were performed to achieve the highest possible signal. Final mass spectrometry parameters were: capillary voltage = 0.30 kV, extractor voltage = 3 V source temperature = 150°C, desolvation temperature =650°C,

desolvation gas flow = 400 L/hr, and collision gas flow 0.15 mL/min. Cone voltages and collision energy were 32 and 38 for analytes with m/z, 513.35 →> 209.30 transition and 32 and 18 for analytes with m/z, 513.35 >→ 335.35 transition, respectively; and 38, and 44 for the internal standard.

Preparation of standards, quality controls, and IS solutions

Sub-stock and working stock solutions of PF-5190457 and IS were prepared using 50% acetonitrile (ACN) and were stored at 4 °C. Standards and quality controls (QCs) samples were prepared by spiking rat or human plasma or rat brain homogenate to achieve desired PF-5190457 concentrations while keeping the organic solvent ≤ 5% of total volume. Standard concentrations of PF-5190457 in rat brain homogenates before extraction were: 0.15, 0.30, 0.68, 2.40, 4.80, 9.60, 19.20, and 24.00 μg/L; QCs concentrations were 0.45, 3.00, and 18.00 μg/L for LQC, MQC, and HQC, respectively. The final standard and QCs concentrations in brain samples are shown in Tables 1 and 2.

Plasma standard concentrations before extraction were 1, 2, 10, 100, 250, 500, 800 and 1000 μg/L; QCs concentrations were 3, 200, 750 μg/L for LQC, MQC, and HQC, respectively. Working internal standard solutions (WIS) composed of 0.1% formic acid in ACN at concentrations of 5 and 10μg/L were used as precipitating solvents for brain and plasma samples, respectively. The final standard and QCs concentrations in plasma samples are shown in Tables 1 and 2.

Protein precipitation and sample extraction

A. Rat brain samples

Brain segments from each rat were weighed individually and homogenized manually on ice using a glass tissue homogenizer with four-fold volume of de-ionized water (w:v) until a homogenous mixture was formed. One part brain homogenate of control blank, standards, QCs, and samples was extracted with two parts of 5 μg/L WIS in 1.5 mL Eppendorf tubes. Double blank samples were extracted with 100% ACN. After vortex mixing for 10 seconds, samples were centrifuged at 5000 xg for 5 min and 10 μL of supernatant was injected onto LC-MS/MS.

B. Rat and human plasma samples

One part of rat or human plasma as control blank, standards, QCs, and plasma samples was mixed with four parts of 10 ng/mL WIS in a 1.5 mL microfuge tube. Double blank samples were extracted with 100% ACN. After vortex mixing for 10 seconds, samples were centrifuged at 5000 xg for 5 min and 5μL of the supernatant was injected onto LC-MS/MS.

Assay validation

Standards and QCs

The method was validated in accordance with the current version of the Food and Drug Administration (FDA) guidance for industry bioanalytical method validation [14]. Calibration curves were constructed by plotting analyte/IS peak area ratio against the nominal concentration of analytes and fitted using a (1/x) weighting method. Accuracy and precision of the assay were determined using three different batches of brain or plasma that were spiked with working stock solutions to achieve standards and QCs concentrations (6 replicate) and extracted as described in the sample

extraction section.

Sensitivity and selectivity

Lower limit of quantification (LLOQ) was determined by concentrations that had % bias ≤ ± 20%, coefficient of variation (CV) ≤ ±20% and signal to noise ratio (S/N) ≤10. Acceptance criteria for QCs (LQC, MQC and HQC) was %bias ≤ ±15%and CV ≤ ±15%. Selectivity assessed by inspecting the presence of noise or peaks at analyte and IS elution time on chromatograms represented blank brain or plasma samples (from 6 subjects).

Stability

Stability of PF-5190457 was investigated by quantifying QC1 and QC3 concentrations in three replicates. Freeze and thaw (three freeze and thaw cycles), bench-top, and short-term stability for up to one month were investigated. Auto-sampler stability was assessed, by re-injecting one of the validation batches kept in the auto-sampler for over 72 hours.

Matrix effect and recovery

Possible interference of matrix effect (ME) in brain and plasma samples was inspected visually through two ways. *First*, possible interference of matrices components was visually inspected on chromatograms generated using post-column infusion [15]. The test was performed by continuously infusing, after the column via a Tee connection, 98% ACN solution (represents the composition of mobile phase at elution time) containing PF-5190457 and IS at highest standards concentrations at a flow rate of 10 µL/min. Simultaneously, extracted blank brain samples, plasma samples, and neat

solution (%50 ACN) were injected using the pre-established LC method. Chromatograms obtained from injecting blank brain or plasma samples were compared with a chromatogram that represented neat solution chromatograms for any signs of suppression and/or enhancement at analyte and IS elution region. *Second*, possible co-elution of analytes and IS with PL was also checked [16,17]. By including MRM transitions of abundant phospholipids (PL) in MS method, we were able to visually locate PL elution region at early stages of method development. Co-elution was avoided by manipulating liquid chromatography conditions and mobile phase gradients.

To determine recovery, two sets of QCs (form six subjects) were prepared. The first set of QCs was prepared in either brain or plasma and was extracted as prescribed in the samples extraction section (pre-extracted matrices QCs). The second set was prepared by spiking extracted blank matrices with standard working solutions to achieve the same final concentration as the concentration in the first set. The percentage ratio of mean peak areas of pre-extracted samples to mean post-extracted spiked samples was used to calculate recovery.

Results and discussion

Sensitivity and selectivity

Brain concentration of analyte was expected to be very low compared to plasma. Therefore, mass spectrometry and chromatographic conditions were optimized using extracted brain samples to improve lower limit of quantification. Adequate sensitivity and selectivity were obtained using Acquity UPLC BEH C18 column. The final UPLC and mass spectrometry parameters were appropriate to set LLOQs at 0.75 and 1 μg/L

for brain and plasma, respectively (**Figure 4**). Chromatograms obtained from pooled blank samples from six subjects and blank neat solutions (50% ACN) were visually inspected and compared for any peaks or noises at elution regions. No sign of interference was noticed. No carryover was detected when double blank samples were injected following the highest calibration concentration.

Curve fitting of the standard curve was comprised of 1/x weighted least squares linear regression. The average correlation coefficient (r^2) of the three validation batches was 0.999. The inter-run % bias and coefficient of variation (CV) were in the recommended limit of ±20 for LLOQ and ±15 for QCs (Table 2).

Stability

Bench top, freeze and thaw, auto-sampler, and short-term storage at –80 C^o for up to four weeks were studied (Table 3). No stability problems were noticed and analytes were stable in extracted matrices for up to 72hrs.

Recovery and matrix effect

Samples processing and extraction procedures showed excellent recovery. The recovery ranged from 102-118% with CV less than 6% for all matrices (Table 3). Endogenous components in biological fluids may interfere and compete for ionization with the analytes of interest [15]. The ME could be either ionization suppression or enhancement, both of which can potentially compromise the integrity of the data [16]. A post-column infusion technique was utilized to examine possible interference of components present in matrices of interest. **Figure 5** shows a representative composite of PF-5190457 and IS traces obtained from post-column infusion at a concentration of

1 µg/mL overlaid on chromatograms obtained from injecting samples. An area of ionization suppression was seen around 0.25 minute in chromatogram from all matrices; slight ionization enhancement was also seen around 0.5 minute in all matrices (**Figure 5**). There was no sign of ionization suppression or enhancement at the retention time of analyte or IS.

The ME was investigated visually first by detecting elution regions of PL components of rat brain, rat plasma and human plasma. MRM of transitions of most common PLs [16,17] were added to the mass spectrometry method. Mass transitions of PLs include m/z, 496 → 184, 520 → 184, 522 → 184, 524 → 184, 758 → 184, 782 → 184. As shown in **Figure 6**, the investigated PLs eluted far enough after analytes of interest in rat brain (A), rat plasma (B) and human plasma (C). It must be noted that PLs's that have m/z of 524 are more abundant in the brain when compared to rat and human plasma. In contrast, PLs's with m/z of 522 seem to be more abundant in rat and human plasma than in rat brain. Since the dilution factors (15 and 5 times for brain and plasma, respectively) and final water proportion in each final matrix extract was different, direct quantitative comparison was not possible.

Assay application

The assay was successfully utilized to measure compound concentrations in rat brains and plasma after administration of PF-5190457 as well as preliminary pharmacokinetic studies in human plasma conducted in the context of phase 1b study. Appropriate approvals were granted by the appropriate NIH Institutional Animal Care and Use Committee (IACUC) and the Institutional Review Board (IRB). **Figure 7** depicts a concentration-time profile of PF-5190457 in a representative human subject

at steady-state after administration of 50 and 100 mg oral dose of PF-5190457.

Conclusion

This is the first reported analytical method for quantification of PF-5190457 in rat brain, rat plasma and human plasma. This LC-MS/MS method was developed and validated in accordance with the current FDA guideline and showed high sensitivity, selectivity and robustness. Simple extraction processes with excellent recovery and sufficient sample cleanness was used. The method allowed us to examine the presence and describe relative components and elution behaviors of the investigated PLs species. The assays were successfully applied for quantification of PF-5190457 in both pre-clinical and clinical studies.

Table 6-1. Summary of standards curve parameters from three individual runs.

Table 1. Summary of standards and calibration curve parameters from three individual runs										
		STD 1	**STD 2**	**STD 3**	**STD 4**	**STD 5**	**STD 6**	**STD 7**	**STD 8**	**R2**
Matrix	**Nominal conc. (µg/L)**	**0.75**	**1.50**	**4.50**	**12.00**	**24.00**	**48.00**	**96.00**	**120.00**	
Rat brain	Mean	0.83	1.47	4.04	11.86	23.47	44.59	97.84	117.85	0.9989
	SD	0.06	0.12	0.63	0.60	0.96	2.91	7.99	7.53	
	%Bias	10.20	-1.92	-10.33	-1.14	-2.20	-7.10	1.91	-1.79	
	CV	7.60	7.87	15.73	5.03	4.08	6.53	8.17	6.39	
	Nominal conc. (ng/mL)	**1.00**	**2.00**	**10.00**	**100.00**	**250.00**	**500.00**	**800.00**	**1000.00**	
Rat plasma	Mean	1.00	2.06	9.95	103.18	258.39	500.11	795.00	999.22	0.9999
	SD	0.10	0.27	1.11	12.63	33.70	23.88	35.87	33.04	
	%Bias	0.13	3.23	-0.47	3.18	3.36	0.02	-0.63	-0.08	
	CV	10.31	12.85	11.19	12.24	13.04	4.78	4.51	3.31	
	Nominal conc. (ng/mL)	**1.00**	**2.00**	**10.00**	**100.00**	**250.00**	**500.00**	**800.00**	**1000.00**	
Human plasma	Mean	0.98	1.89	9.42	99.79	251.93	476.21	831.43	993.69	0.9989
	SD	0.09	0.25	3.89	11.29	14.59	30.72	38.45	28.31	
	%Bias	-2.12	-5.51	-5.81	-0.21	0.77	-4.76	3.93	-0.63	
	CV	9.05	12.99	41.25	11.32	5.79	6.45	4.62	2.85	

***n* = 3 (1 replicate for each of the three validation runs). %Bias calculated as 100 × (mean-nominal)/nominal. CV calculated as 100 × std dev/mean.**

Table 6-2. Summary of quality control samples from three individual runs

Table 2. Summary of quality control samples from three individual runs.

		LLOQ	QC1	QC2	QC3
Matrix	**Nominal conc. (µg/L)**	**0.75**	**2.25**	**15.00**	**90.00**
Rat brain	Inter-run Mean	0.77	2.28	14.51	96.95
	Inter-run SD	0.10	0.24	1.14	5.47
	Inter-run %Bias	2.86	1.46	-3.25	7.72
	Inter-run CV	12.64	10.40	7.84	5.65
Rat plasma	**Nominal conc. (ng/mL)**	**1.00**	**3.00**	**200.00**	**750.00**
	Inter-run Mean	1.00	3.12	214.9	786.99
	Inter-run SD	0.17	0.35	18.30	57.02
	Inter-run %Bias	0.21	4.12	7.40	4.93
	Inter-run CV	16.99	11.17	8.50	7.24
Human plasma	**Nominal conc. (ng/mL)**	**1.00**	**3.00**	**200.00**	**750.00**
	Inter-run Mean	0.94	2.96	205.45	758.24
	Inter-run SD	0.15	0.40	13.99	83.22
	Inter-run %Bias	-5.67	-1.17	2.73	1.10
	Inter-run CV	15.55	13.63	6.81	10.97

n = 18 (6 replicates for each validation run). %Bias calculated as 100 × (mean-nominal)/nominal. CV calculated as 100 × std dev/mean.

Table 6-3. Results of stability studies

Table 3 . Results of stability studies

Matrix	QCs		Bench top 12 hrs	Freeze & thaw	Auto-sampler 72 hrs	Short term 1 weeks	Recovery %
Rat brain	QC1 (2.25) (ng/g)	Mean	2.3	2.2	2.4	2.5	102.0
		Bias %	2.2	-2.2	6.6	11.1	–
		CV	4.3	9.5	6.4	6.1	-1.2
	QC3 (90.0) (ng/g)	Mean	77.2	82.1	83.6	79.4	115.0
		Bias %	-14.2	-8.8	-7.1	-11.8	–
		CV	6.2	4.5	11.6	5.8	-1.4
Rat plasma	QC1 (3.0) (ng/mL)	Mean	3.4	3.1	3.0	3.1	117.0
		Bias %	14.4	3.3	1.2	3.3	–
		CV	11.0	8.5	11.5	5.6	-1.6
	QC3 (750) (ng/mL)	Mean	709.5	826.2	757.5	765.5	104.0
		Bias %	-5.4	10.2	1.0	2.1	-1.3
		CV	4.0	4.4	10.6	6.5	
Human plasma	QC1 (3.0) (ng/mL)	Mean	3.4	3.3	2.9	3.4	114.0
		Bias %	13.3	10.0	-3.2	13.3	–
		CV	6.1	6.1	9.4	7.8	-5.5
	QC3 (750) (ng/mL)	Mean)	797.3	826.5	801.2	800.2	118.0
		Bias %	6.3	10.2	6.8	6.7	–
		CV	2.5	2.8	11.1	1.8	-1.4

n = 3 . %Bias calculated as 100 × (mean-nominal)/nominal.
CV calculated as 100 × std dev/mean.

Figure 6-1. Q1 scan of PF-5190457 shows the abundant adducts, $[M+H]^+$ and $[M+H_4]^+$.

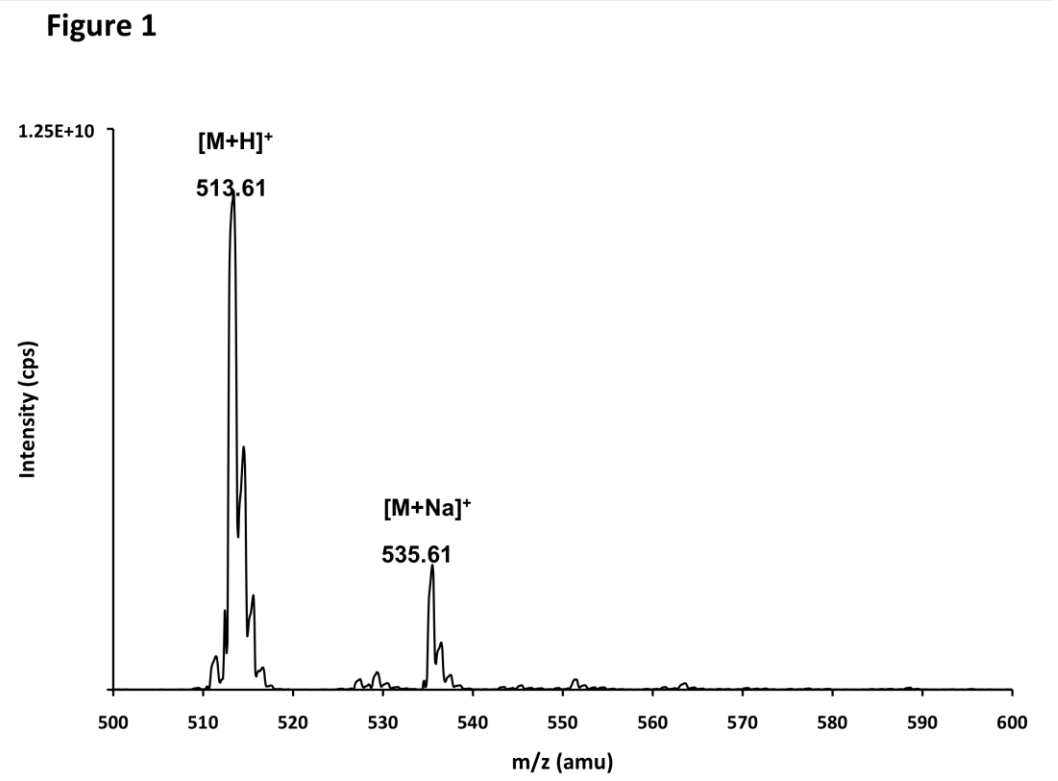


Figure 6-2. Q3 scan shows fragmentation pattern of PF-5190457 $[M+H]^+$ and intensity of daughter ions.

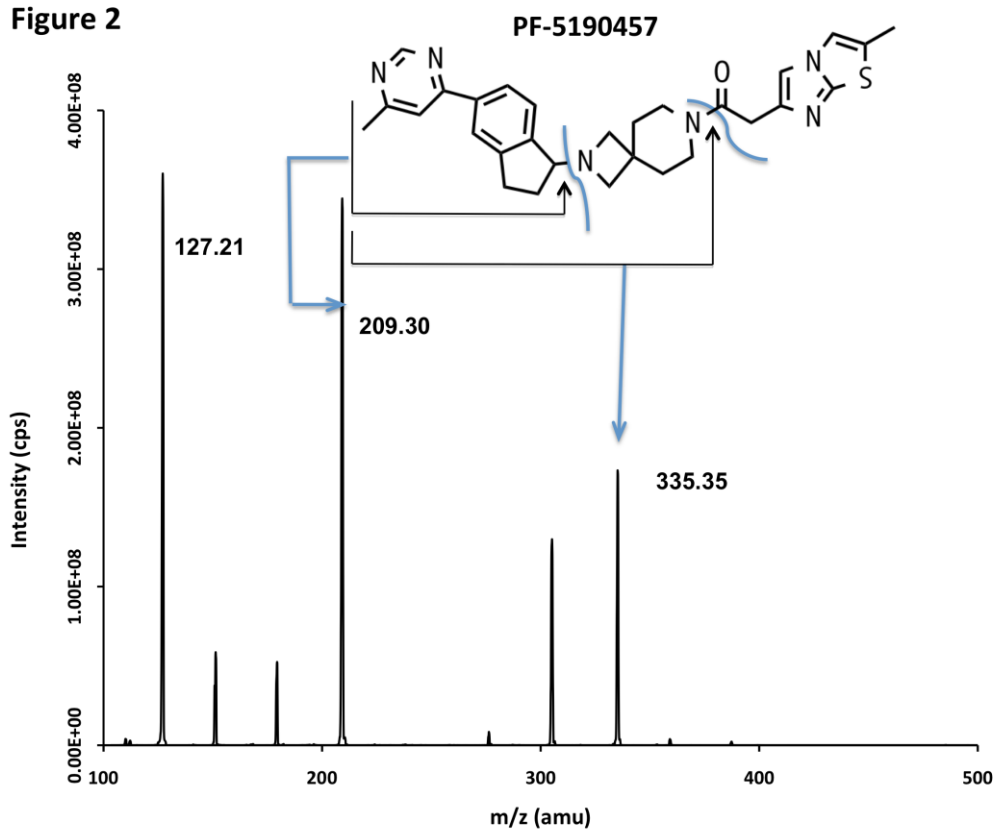


Figure 6-3. Chromatograms of ghrelin antagonist (PF-5190457) (A, B, and C) and the internal standard) at LLO Q (D,E and F) and in rat brain, rat plasma and human plasma samples, respectively.

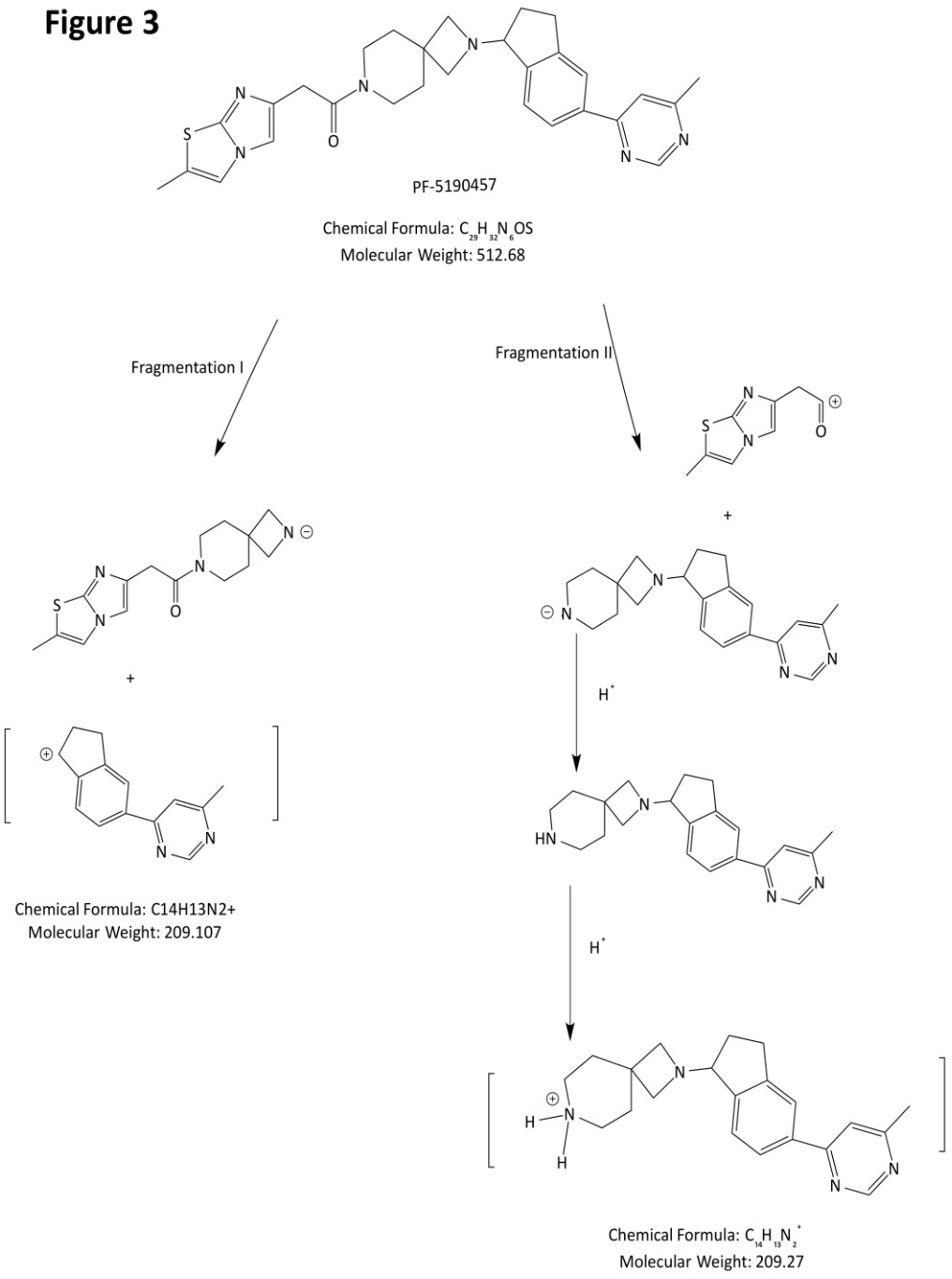
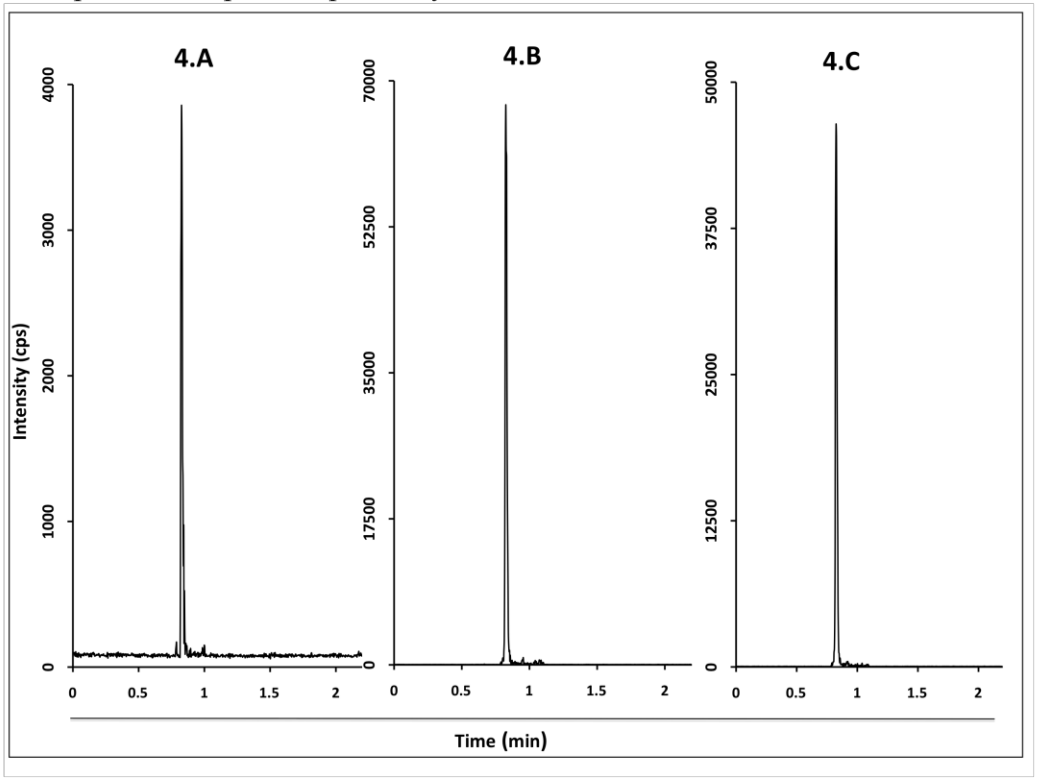


Figure 6-4. Chromatograms of ghrelin antagonist (PF-5190457) (A, B, and C) and the internal standard) at LLO Q (D,E and F) and in rat brain, rat plasma and human plasma samples, respectively.



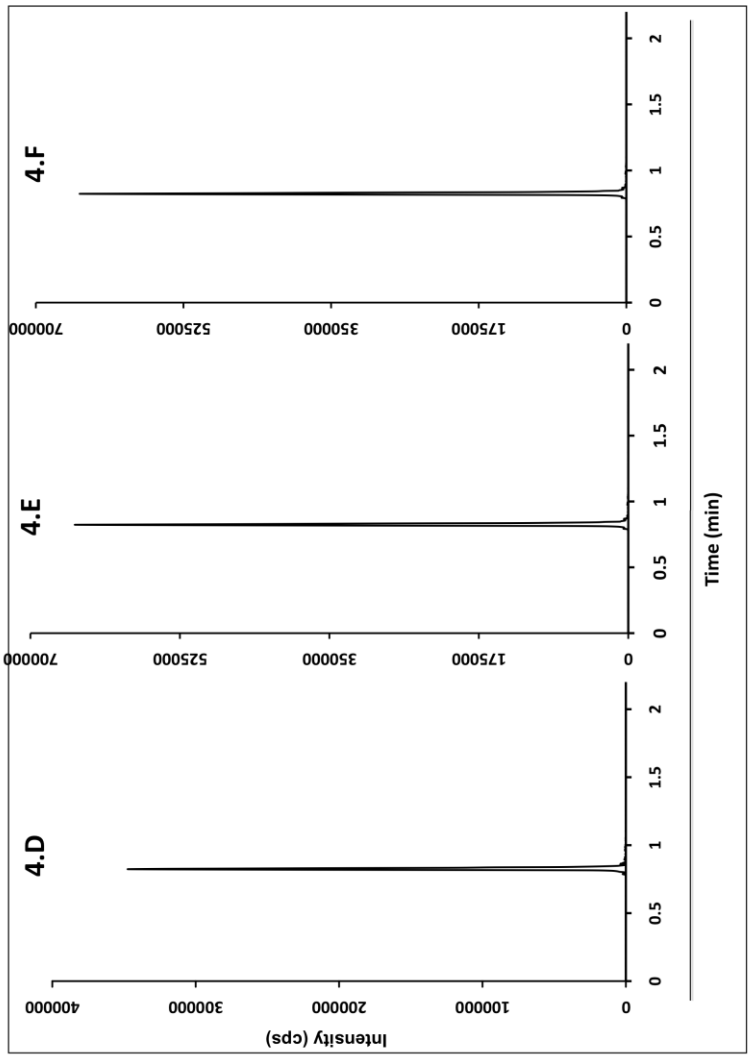
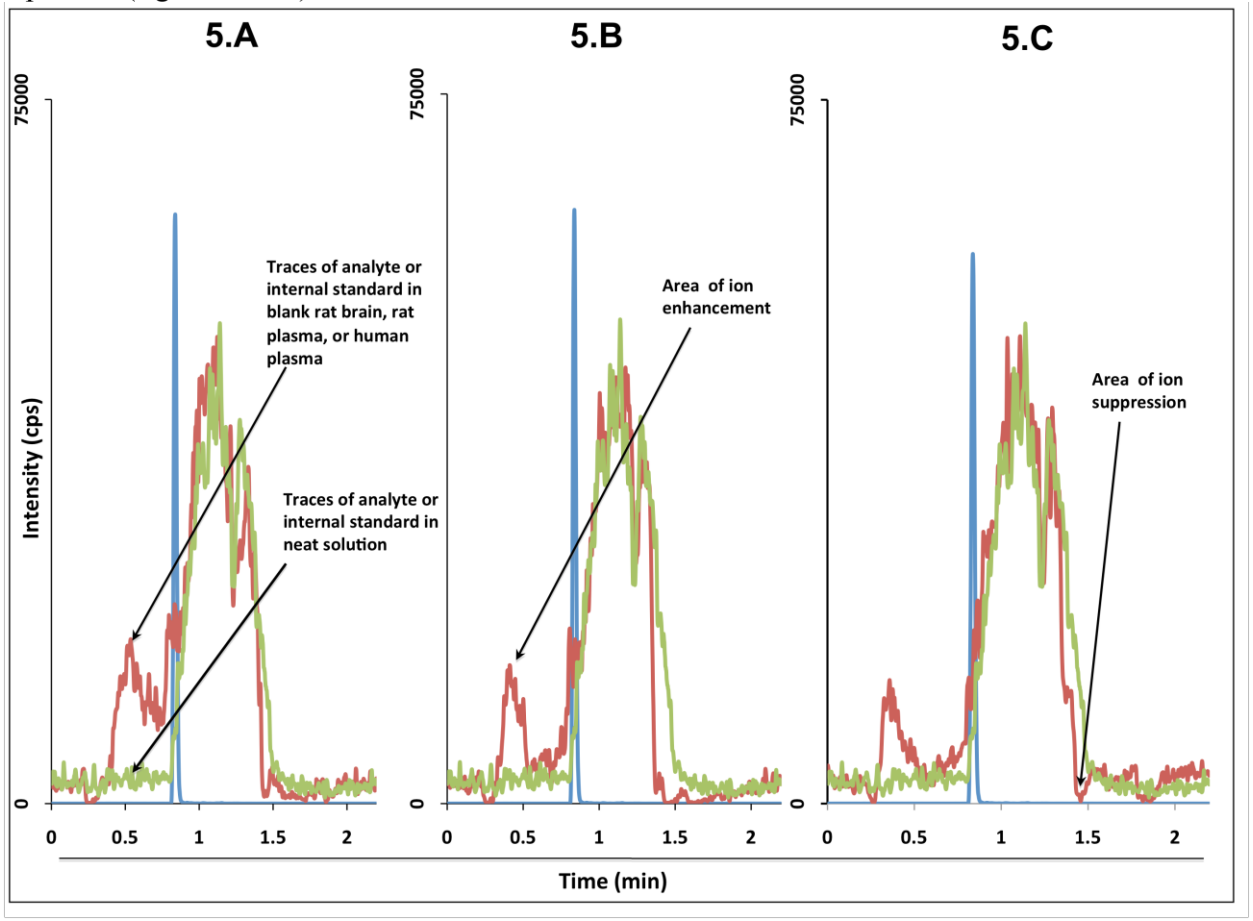

4.D
4.E
4.F
Intensity (cps)
Time (min)
0
100000
200000
300000
400000
175000
350000
525000
700000
0.5
1
1.5
2

Figure 6-5. A composite chromatogram of traces obtained from continues post-column infusion chromatograms of PF-5190457 (A, B, and C) and the internal standard (D, E, and F) overlaid on a chromatograms of injections of rat brain (left column), rat plasma (middle column) and human plasma (right column).



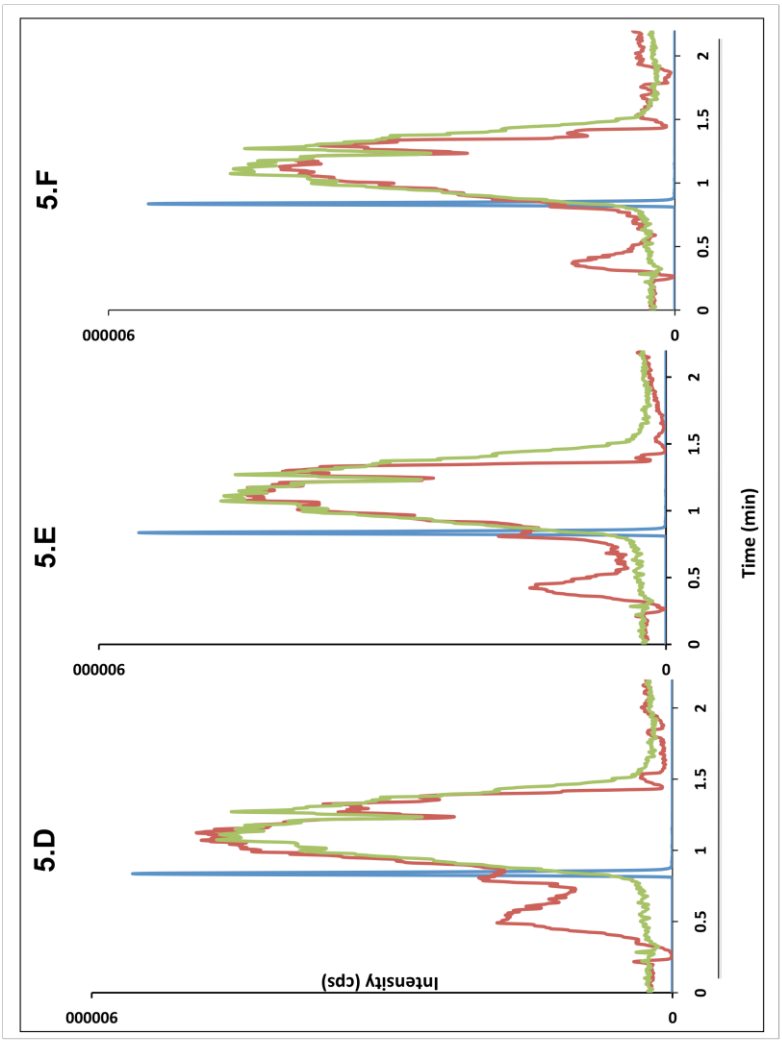
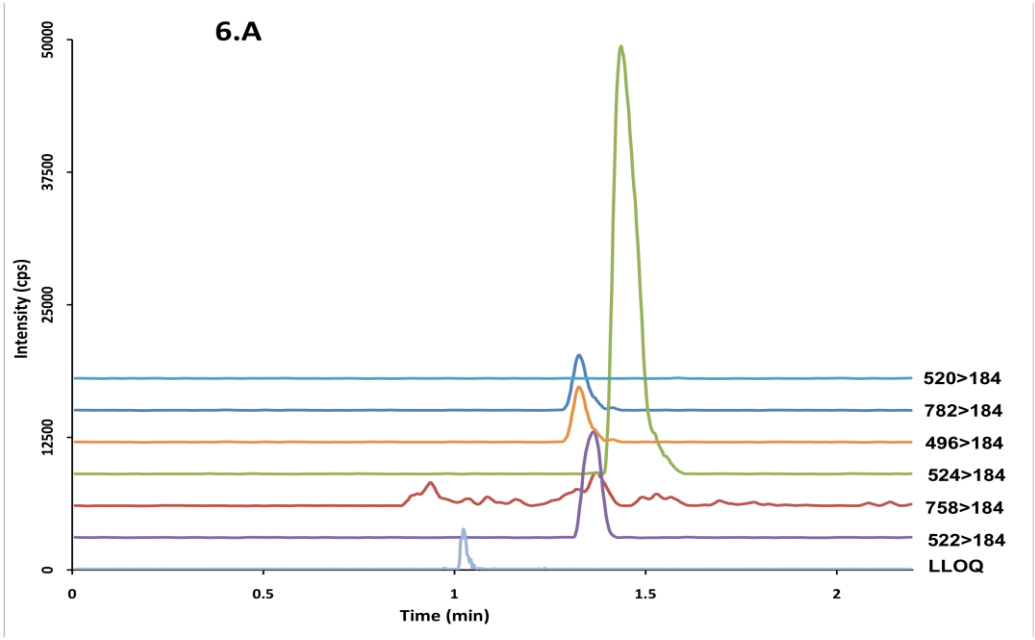
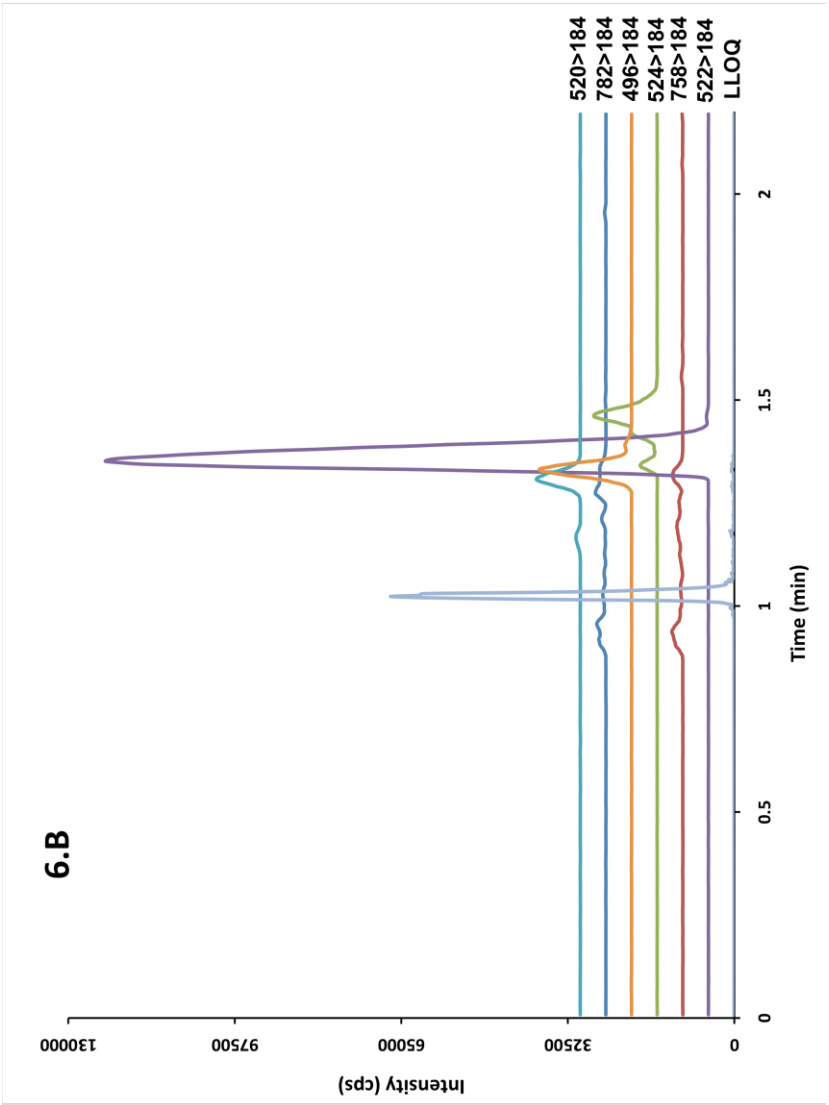

5.D
5.E
5.F
Intensity (cps)
Time (min)
900000
0
0.5
1
1.5
2

Figure 6-6 Chromatograms depicting traces of phospholipids obtained from injecting pooled blank samples of rat brain (A), rat plasma (B) and human plasma (C). MRM transition of each individual phospholipids species is shown on the right side of the graph. The figures show the relative amount of PF-5190457 to PLs in each matrix.




6.B
Intensity (cps)
0
32500
65000
97500
130000
Time (min)
0
0.5
1
1.5
2
520>184
782>184
496>184
524>184
758>184
522>184
LLOQ

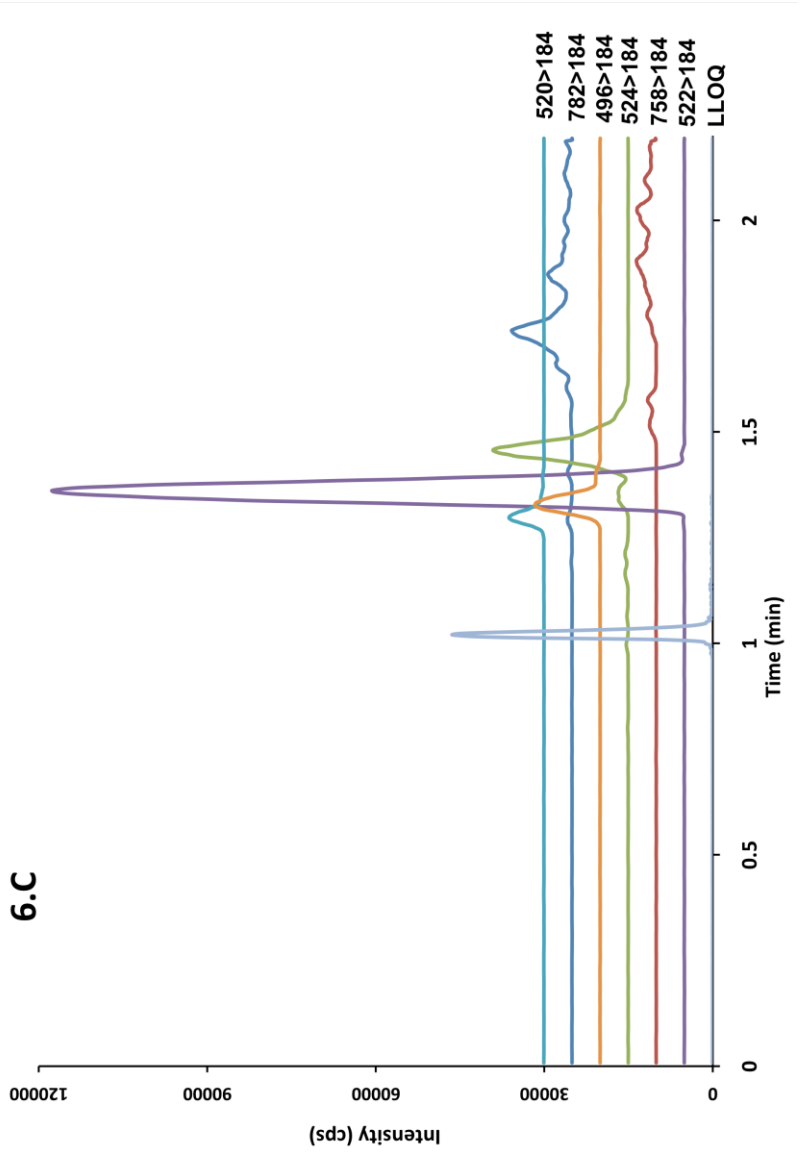
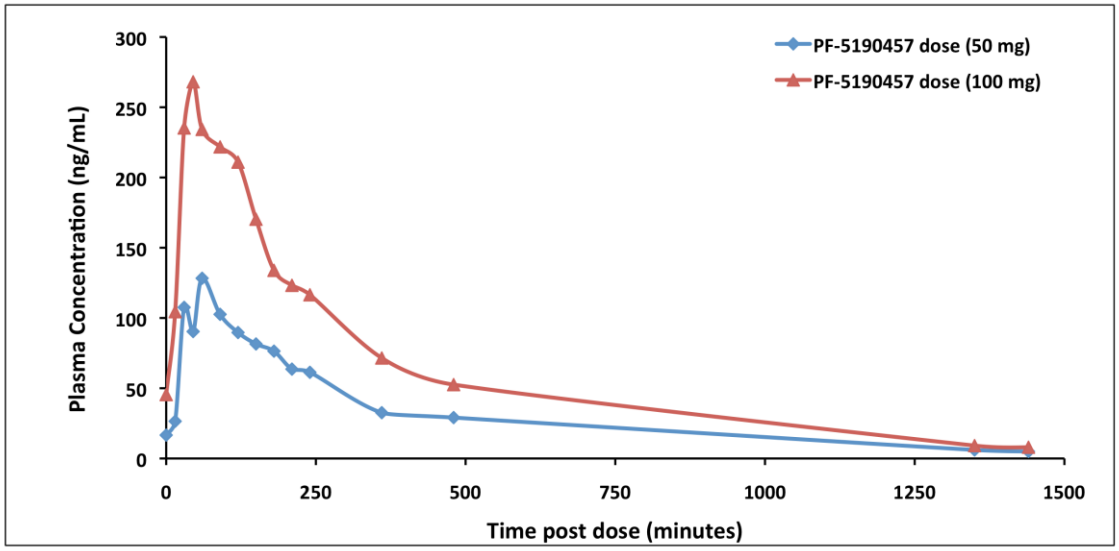

6.C
Intensity (cps)
0
30000
60000
90000
120000
Time (min)
0
0.5
1
1.5
2
520>184
782>184
496>184
524>184
758>184
522>184
LLOQ

Figure 6-7 Concentration-time profiles of PF-5190457 in a representative study volunteer after ingestion of 50 and 100 mg doses of PF-5190457 by oral route.



References

1. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*, 402(6762), 656-660 (1999).

2. Tong J, Prigeon RL, Davis HW *et al*. Ghrelin suppresses glucose-stimulated insulin secretion and deteriorates glucose tolerance in healthy humans. *Diabetes*, 59(9), 2145-2151 (2010).

3. Solomon A, De Fanti BA, Martinez JA. Peripheral ghrelin participates in glucostatic feeding mechanisms and in the anorexigenic signalling mediated by CART and CRF neurons. *Nutritional neuroscience*, 8(5-6), 287-295 (2005).

4. Druce MR, Wren AM, Park AJ *et al*. Ghrelin increases food intake in obese as well as lean subjects. *International journal of obesity*, 29(9), 1130-1136 (2005).

5. Neary NM, Small CJ, Wren AM *et al*. Ghrelin increases energy intake in cancer patients with impaired appetite: acute, randomized, placebo-controlled trial. *The Journal of clinical endocrinology and metabolism*, 89(6), 2832-2836 (2004).

6. Kirchner H, Tong J, Tschop MH, Pfluger PT. Ghrelin and PYY in the regulation of energy balance and metabolism: lessons from mouse mutants. *American journal of physiology*. *Endocrinology and metabolism*, 298(5), E909-919 (2010).

7. Volkow ND, Wang GJ, Tomasi D, Baler RD. The addictive dimensionality of obesity. *Biological psychiatry*, 73(9), 811-818 (2013).

8. Leggio L, Addolorato G, Cippitelli A, Jerlhag E, Kampov-Polevoy AB, Swift RM. Role of feeding-related pathways in alcohol dependence: A focus on sweet preference, NPY, and ghrelin. *Alcoholism, clinical and experimental research*, 35(2), 194-202 (2011).

9. Leggio L. Role of the ghrelin system in alcoholism: Acting on the growth hormone secretagogue receptor to treat alcohol-related diseases. *Drug news & perspectives*, 23(3), 157-166 (2010).

10. Jerlhag E, Egecioglu E, Landgren S *et al*. Requirement of central ghrelin signaling for alcohol reward. *Proceedings of the National Academy of Sciences of the United States of America*, 106(27), 11318-11323 (2009).

11. Leggio L, Ferrulli A, Cardone S *et al*. Ghrelin system in alcohol-dependent subjects: role of plasma ghrelin levels in alcohol drinking and craving. *Addiction biology*, 17(2), 452-464 (2012).

12. Leggio L, Zywiak WH, Fricchione SR *et al*. Intravenous Ghrelin Administration Increases Alcohol Craving in Alcohol-Dependent Heavy Drinkers: A Preliminary Investigation. *Biological psychiatry*, 76(9), 734-741 (2014).

13. Bhattacharya SK, Andrews K, Beveridge R *et al*. Discovery of PF-5190457, a Potent, Selective, and Orally Bioavailable Ghrelin Receptor Inverse Agonist Clinical Candidate. *ACS medicinal chemistry letters*, 5(5), 474-479 (2014).

14. US Department of Health and Human Services. Food and Drug Administration (FDA). Center for Drug Evaluation and Research (CDER). Guidance for Industry. Bioanalytical Method Validation (2001).

15. Dams R, Huestis MA, Lambert WE, Murphy CM. Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: influence of ionization type, sample preparation, and biofluid. *J. Am. Soc. Mass Spectrom.*, 14(11), 1290-1294 (2003).

16. Chambers E, Wagrowski-Diehl DM, Lu Z, Mazzeo JR. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 852(1-2), 22-34 (2007).

17. Zhang G, Wujcik CE. Overcoming ionization effects through chromatography: a case study for the ESI-LC-MS/MS quantitation of a hydrophobic therapeutic agent in human serum using a stable-label internal standard. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 877(22), 2003-2010 (2009).