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Alternative matrices for therapeutic drug monitoring of immunosuppressive agents using LC–MS/MS

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Abstract

Immunosuppressive drugs used in solid organ transplants typically have narrow therapeutic windows and high intra- and intersubject variability. To ensure satisfactory exposure, therapeutic drug monitoring (TDM) plays a pivotal role in any successful posttransplant 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 nonconventional matrices, namely oral fluids, fingerprick blood and intracellular and intratissue 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.

Therapeutic drug monitoring (TDM) is an integral part of immunosuppressive therapy following organ transplantation because of the narrow therapeutic index and high inter- and intrasubject 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 underdosed 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.

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Use of 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 matrices, 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 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 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 HPLC with MS. Three atmospheric pressure ionization, namely, electrospray ionization, atmospheric-pressure chemical ionization, and atmospheric-pressure photo-ionization 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 is most commonly used in quantifying polar to ionic compounds, and in metabolic 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 IS [21] and chromatographic separation of analytes from regions of ion enhancement or suppression can mitigate/eliminate the effect of ME [28].

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]. 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] (See Figure 1). 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 2 and Table 1, respectively.

Recently, a Saliva Excretion Classification System has been proposed to predict the ability of drugs to diffuse into the OF [57,58]. 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.

Oral fluid collection techniques & storage 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 reabsorption and secretion [29,48]. Stimulation may alter the salivary composition and pH [59], thereby may affect the partitioning of drugs between the OF and plasma [60] 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 nonionized 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]. Using Henderson Hasselbalch 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.

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 [61]. In a study reported by Groschl *et al.* [61], 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, Nümbrecht, Germany) 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 [62]. 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 [63]. To prevent nonspecific 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 [61,64–65].

Sample preparation & extraction

The mucopolysaccharide content of OF may interfere with the accuracy of pipetting [66]. 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 [66]. Simple preanalysis 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].

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 [66]. In contrast, the validity of salivary testosterone measurements can be compromised by even minimal blood contamination from microinjuries caused by routine teeth brushing, as detected by the transferrin immunoassay (Salimetrics LLC, State College, PA, USA) [66]. 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 nonfasting 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.

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.

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 nonspecifically 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 radiolabeled 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 [67], drugs that modulate the lipid profile [44,68], nutritional status [67] and clinical conditions [49,67]. 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 [67]. The outcome of immunosuppressant therapy with CsA is improved by a higher free fraction percentage [69].

There is a high variability in the free fraction of CsA with a mean $\pm$ SD of $1.53 \pm 0.38\%$ in the lung and heart transplant recipients [44] and a range from 0.5 to 4.2% [49]. 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).

Tacrolimus

Tacrolimus is a highly lipophilic compound (Table 1) 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 [43,70]. There is only one published method for the utilization of the OF matrix for TAC quantification [24] (Table 2).

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 ($\log D$ 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 [25,38,71]. Therefore, the chromatographic separation of MPA and MPAG peaks is necessary to avoid overestimating the parent drug concentration.

Prednisolone

Prednisolone (PLN) is a synthetic glucocorticoid with an unspecific mechanism of action [72]. Prednisolone is widely prescribed as a part of immunosuppressive therapy regimens in solid organ transplantation [73]. The free fraction of PLN increases in certain clinical conditions such as diabetes [52]. In addition, the free fraction is dose-dependent and exhibits circadian variability (approximately 22% higher in the morning) [74]. The PLN plasma unbound fraction demonstrates a high correlation with the salivary level and a lower correlation with the concentration of the prodrug prednisone (PN) [75,76]. Total and free concentrations of PLN+PN in the OF and plasma display an excellent association [76] (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 noninvasive alternative to venous blood sampling if a good correlation between total blood and OF drug concentrations is established.

DBS & liquid fingerprick blood sampling

DBS and liquid fingerprick blood (LFB) sampling are other techniques that have benefited from the introduction of LC–MS/MS [12–14,77–83]. The first report of the use of fingertip blood to measure an immunosuppressive agent was published in the late 1980s [84]. The radioimmunoassay (RIA) technique was utilized to quantify CsA in 20- μ l blood samples obtained from the fingertips of renal transplant recipients with a lower limit of quantification (LLOQ) of 62.5 μ g/l. Fingerprick sampling is much less invasive than venipuncture and offers the possibility of home self-sampling at the patient's convenience [85]. However, adequate patient training might be needed for optimum sample collection [86]. Additionally, proper sample handling and storing after collection are required to avoid deterioration and to ensure stability during mailing and transportation [79,85].

Sample collection

After cleaning the fingertip with a suitable disinfectant [11,14,85,87], a small laceration is made using spring-loaded lancets that are designed to minimize pain and discomfort [79,83,85,88]. A fingerprick blood sample is processed either as a DBS (Supplementary Table 1) or in liquid form (LFB) (Table 3).

In the DBS technique, blood samples are either applied directly from the fingertip after discarding the first drop [77,79–80,82,85,88]; 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 [15,83,89]. 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]. LFB 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].

Extraction procedure & recovery of DBS & 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 [15,81,88] to ultrasonication at temperatures of up to 80°C [77–78,83,89]. The different pretreatment conditions used for the samples result in significant differences in the final yield (Supplementary Table 1). The applied blood volume, card type, punched area and HT may also play important roles in the extraction recovery and method reproducibility [90,91]. Therefore, these variables should be examined for the analyte of interest, and corrections should be applied if necessary and feasible [92]. LFB samples are pretreated with a zinc sulfate solution (0.1–0.4 mol/l) followed by protein precipitation with ACN and centrifugation [11–14].

Effect of blood volume

A good precision of the estimation of CsA, SIR and TAC ($CV = 4.3\text{--}13.5\%$) was produced using a 25–100 μl blood drop on a Whatman 903 card with an 8-mm punch size [81]. A different study [93] reported that 20 μl of blood was enough to fill the designated area on the Whatman 903 card. In contrast, another study [77] used the same type of filter paper and punch size reported that drops with a volume of 20 μl were insufficient to fill the predetermined area. The discrepancy between the two studies could be attributed to differences in the HT values of the blood samples used.

Influence of the type of sampling card

A Whatman Protein saver 903 card (LifeSciences GH) [94] is the most commonly used card for immunosuppressive drug testing (Supplementary Table 1). This card is additive-free and made from 100% pure cotton linters [95]. Whatman FTA and FTA Elute are high quality papers that are chemically treated to provide cell lysis, protein denaturation and prevention of microorganism growth [95]. 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 [94]. 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 [96].

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 [92]. Heinig *et al.* [93] 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 ($CV = 17\text{--}26\%$) 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.

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 [91]. 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 [93]. 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) [93]. Consistency of the punching location helps to improve the reproducibility [93] and application of a larger spot than the size of the punched disc ensures sampling from the center of the spot [77].

Effect of hematocrit

Normal HT values range from 42 to 52% in males and from 37 to 48% in females [97]. Samples from patients with a high HT create drops that are more viscous and have smaller volumes [98]. 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 [92]. Consequently, the

concentration of certain analytes can be overestimated [83,91–92,98]. A high HT has been reported to increase the MPA content by approximately 10% [98]. Similar findings have been reported for CsA from blood samples with high HT values (0.72%), demonstrating an approximately 10–14% higher CsA concentration [83]. 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 [77,83,97–98]. 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 with less than 85% for non-normalized HT values [77]. The effect of HT on the recovery of EVE, SIR and TAC appears to be minimal [77].

Recently, a new technique was proposed to overcome variability in volumes of blood samples applied to filter paper, which arise from differences in HT value [99,100]. It utilizes simple and practical procedures using a volumetric absorptive micro sampler device (VAMS). This consists of a porous absorbent polymeric tip capable of absorbing 10 µl of blood more precisely, utilizing capillary force.

Matrix effect

The extracted matrix from DBS appears to have a negligible effect on ME [77,82–83,88,93]. 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 [93]. However, remains were further reduced after proper sample cleaning using SPE [93]. 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 [92]. The ME effect on CsA was diminished when a deuterated IS was used.

Stability

Despite the use of the same DBS collection paper (Whatman 903), a discrepancy in stability has been reported, especially for CsA (Supplementary Table 1). 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 2 h, and the CsA was extracted from a 4-mm disc. Samples collected with capillary devices with or without EDTA were stable for up to 12 h at 8 and 20°C; the concentration decreased significantly by 24 h. In contrast, no identifiable changes in the blood samples processed in liquid form were observed. Shorter stability time in the DBS samples compared with the capillary blood samples, may indicate an insufficient drying time (2 h) and/or poor storage conditions and handling [95].

In another study [83], 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 [81]

reported that CsA extracted from an 8-mm disc prepared using 50 μ l EDTA venous blood dried for 3 h 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 [81,83] (see Supplementary Table 1).

Tacrolimus that was measured in EDTA venous blood (50 μ l) applied immediately onto a filter paper and dried at room temperature for 3 h showed stability for up to 5 days at 60°C, and SIR was stable for the same period of time at 37°C [81]. Tacrolimus in fingertip blood samples applied directly onto the filter paper also showed stability for up to 7 days at 37°C [88]. In addition, EVE appeared to be stable for up to 3 days at 60°C and for 32 days at 4°C [82]. 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 [77].

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 [14,85], EVE [82] and CsA [12] DBS samples seemed to be stable during mailing and transportation, supporting LFB and DBS home sampling.

Patient preference

Self-fingerprick sampling is well tolerated with no serious discomfort as reported by children [14,87] or adult transplant patients [12,85,101]. 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 [12,79].

Clinical application of DBS & 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 [82]. Cheung *et al.* [85] used DBS to estimate TAC exposure (AUC_{0-12}) utilizing a limited sampling strategy (C_2 and C_4) in 36 kidney transplant recipients. The DBS 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 with 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 μ g/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 on Intracellular and intratissues concentration below). However, the relative ease of DBS and LFB sampling compared with 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.

Intracellular concentration

Despite maintaining a satisfactory blood level of immunosuppressants through intensive TDM, rejection rates still remain between 8–15% [102], 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 [103–105]. In heart transplant recipients, there is a greater incidence of rejection associated with a higher peripheral blood monocyte cell (PBMC) count [106]. Lymphocytes express P-gp efflux transporter, which is also known as multidrug resistance protein 1 encoded by the *ABCB1* gene [107–109]. This transporter is responsible for moving xenobiotics from the intracellular to the extracellular environment [109]. 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 [109,110]. Both CsA and TAC are well-documented substrates of P-gp [110–112]. *In vitro* data indicate that SIR is a substrate and a weak inhibitor of the P-gp transporter [113–115], while EVE has shown a weak inhibitory effect on P-gp [115]. Higher incidence of rejection is proportionally correlated with higher expression of *ABCB1* gene on PBMCs obtained from heart [106] and liver [116,117] transplant recipients who have been prescribed CsA or TAC. The levels of immunosuppressants in lymphocytes, including CsA [110,118–122], TAC [110–112,119–120,122–130], SIR [131] and EVE [132], have been investigated in solid organ transplant patients (Table 4).

There is a histologically and clinically proven rejection associated with a lower level of TAC in PBMCs measured at day 7 posttransplantation in liver transplant recipients [123]. 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 [112,119]. Contradictory findings have been reported for CsA. A study by Gustafsson *et al.* [125] involving heart transplant recipients co-treated with MPA reported a high correlation ($r^2 = 0.98$, $p < 0.001$) between CsA concentrations in 2 h postdose (C_2) whole blood samples and lymphocyte AUC_{0-12h} exposure (expressed as $ng \times h/10^{-6}$ 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 predose (C_0) samples of blood and intralymphocytes from heart transplant patients was also reported in a recent study by Robertsen *et al.* [118]. 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, $r = 0.09$, $p = 0.71$) [111]. Slightly better correlation was observed in C_0 samples from stable renal, liver and lung transplant recipients ($r = 0.30$, $p < 0.001$) [110]. A study by Falck *et al.* [126] involving kidney transplant recipients reported that, patients who experienced rejection displayed significantly lower CsA intralymphocyte AUC_{0-12h} exposure compared with the nonrejection 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 ($p = 0.014$) 3 days before showing clinical signs of rejection. Regarding EVE, a poor correlation between blood and PBMCs concentrations has been reported ($r = 0.32$) [132]. 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 [106].

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 intralymphocytes at day 7 and steady state [112]. 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 noncarriers. 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 with 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 [110]. Carriers of 3435T showed an increase in intracellular CsA concentrations of 1.7 times ($p = 0.04$) compared with 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 with wild type. The 2677T polymorphism did not affect the intracellular concentration of CsA.

CYP3A-metabolizing enzymes are also expressed in lymphocytes [133,134]. CYP3A enzymes are polymorphic [135–138], but the intracellular TAC concentration is unlikely to be influenced by genetic polymorphisms in CYP3A enzymes [112].

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.

Sample preparation & 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 4). 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 SPE.

Intratissue concentration

Early reports on the measurement of intratissue concentrations of immunosuppressive agents date to the early 1990s [139–141] (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 [118,142–145] (Table 5).

Postmortem examinations have revealed that CsA and its metabolites accumulate rapidly in tissues after administration [139]. 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 [139]. Tissue CsA concentrations were highest in the pancreas, followed by the spleen, liver, kidney, lung and heart. In a recent study [143], 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.* [141] 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 with the rejection group. Moreover, little to no correlation in CsA concentrations has been reported between the blood and the kidney ($r = 0.168$, $p > 0.05$) [143] or endomyocardial biopsies ($r^2 = 0.029$, $p = 0.48$) [118].

Similar findings have been reported for TAC in liver transplant recipients [140]. 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 C₀ blood samples and liver tissues on day 5 and 7 after transplantation [123,142]. 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) [123,142]. Higher concentrations of TAC in liver tissues were associated with lower Banff scores and consequently fewer episodes of rejection [123,142]. In contrast, a poor correlation has been reported between Banff scores and the blood level of TAC ($r^2 = 0.0281$) [123]. In kidney transplant recipients (two patients) [144], a decrease in TAC was observed in tissue and C₀ whole blood over time (16–300 days) but, there was no correlation between the two measurements.

Only one published study investigated the intratissue 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 intratissue concentrations of MPA [145].

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

The intersubject 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 [146]. 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 noncarriers ($p = 0.036$). 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 [146]. In summary, the blood concentration of immunosuppressive drugs in solid organ transplant recipients is a poor predictor of intrahepatocyte levels.

Conclusions & 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 intratissue 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 h, 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 noninvasively 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 [147]. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Papers of special note have been highlighted as:

- of interest;
- of considerable interest

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Key terms

Matrix effect	Interference of matrix components with ionization of the analyte of interest at the ionization site in LC–MS/MS, resulting in ion enhancement or suppression.
Deuterated IS	Isotopic analogue that co-elutes with the analyte of interest and therefore would have similar chromatographical conditions.
Transferrin	Iron-binding plasma protein. A high transferrin level in the oral fluid indicates the presence of traces of blood.
Liquid fingerprick blood	Blood sample collected from the fingertip and processed in liquid form.
P-gp transporter	Transporter responsible for the removing of the xenobiotic from the intracellular environment to the extracellular environment.

Executive summary

LC–MS/MS method development

- The matrix effect is one of the main challenges that must be appropriately addressed.
- The use of a deuterated IS aids in mitigating the matrix effect.
- Proper sample treatment is essential for the development of sensitive reproducible methods.

Oral fluid

- Provides a noninvasive, simple and cheap sample collection approach.
- The drug concentration measured in oral fluid is considered a representative for the free drug fraction and can associate more with clinical outcomes and toxicity.

Dried blood spot

- Compared to venipuncture, it provides a less invasive, simple and cheap sample collection approach.

Intracellular & intratissue drug concentration

- Provides a mean to quantify the drug concentration at the site of action.
- Provides a good prediction of allograft survival and toxicity.

Figure 1. 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

Red discs represent erythrocytes; pink circles represent lymphocytes; blue shapes represent plasma proteins. For color images please see online <http://www.future-science.com/doi/full/10.4155/BIO.15.35>.

Figure 2. Immunosuppressive agents included in this review

Table 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) [‡]	Ref.
Cyclosporine	0.5–4.0	1202.6	C ₆₂ H ₁₁₁ N ₁₁ O ₁₂	3.96	4.09	4.09	11.83	279	[49]
Everolimus	~ 26	958.2	C ₅₃ H ₈₃ NO ₁₄	3.35	4.24	4.24	9.96	205	[50,51]
Mycophenolic acid	1–2.5	320.3	C ₁₇ H ₂₀ O ₆	2.92	2.57	0.76	3.57	93	[4]
Prednisolone	6.3–27	360.4	C ₂₁ H ₂₈ O ₅	1.5	1.66	1.66	12.58	95	[52,53]
Sirolimus	~ 8	914.2	C ₅₁ H ₇₉ NO ₁₃	3.54	4.21	4.21	9.96	195	[54]
Tacrolimus	1.0	804.0	C ₄₄ H ₆₉ NO ₁₂	3.96	4.09	4.09	9.96	178	[3]

[†] PK value for the most acidic functional group.

[‡] Information was obtained from ChemsSpider [55].

[§] Information was obtained from DrugBank [56].

Table 2

Published LC–MS/MS assays for quantification of immunosuppressive drugs in oral fluids (OF).

Drug	Patients	Collection device	Extraction method	Correlation	Chromatographic condition	Precursor ion (m/z) > product ion (m/z)	Ref.
CsA	RTRs (n = 15)	Passive drool	LLE with 94:6 vol/vol ACN; H ₂ O 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 + NH ₄] ⁺ 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 H ₂ O. 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.)	[M + H] ⁺ MPA: 319.0 > 190.8 IS: indomethacin:	[38]
MPA	RTR (n = 9) HV (n = 8)	NR	LLE with equal volume CAN R: 84.1–86.7% IV: 10µl	Total plasma MPA $r = 0.838$. Free plasma MPA $r = 0.816$	Isocratic: 45:55 (v: v) H ₂ O: 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: MPAG: 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]

Drug	Patients	Collection device	Extraction method	Correlation	Chromatographic condition	Precursor ion (<i>m/z</i>) > product ion (<i>m/z</i>)	Ref.
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 ₂ O (A), MeOH (B) both contain 2 mM ammonium acetate/0.1 FA Column: C18 cartridge Run time: NR LLOQ: NR	NR	[24]

ACN: Acetonitrile; Ammonium adduct: [M+NH₄]⁺; CsA: Cyclosporine A; CsC: Cyclosporine C; FA: Formic acid; HV: Healthy volunteers; Ion adduct [M + H]⁺; IV: Injection volume; MeOH: Methanol; MPA: Mycophenolic acid; MPAG: Mycophenolic acid glucuronide; NR: Not reported; R: Recovery; RTR: Renal transplant recipient.

Table 3

Published LC–MS/MS assays for quantification of immunosuppressive drugs in fingerprick samples.

Drug/reference	Subjects	Blood sample volume	Extraction method	Chromatographic conditions	Precursor ion (m/z) > product ion (m/z)	Ref.
CsA	HTRs and LgTRs (n = 65); RTRs (n = 33)	10 μ l	Pretreatment with 0.1 mmol zinc sulfate solution and LLE with CAN IV: 5 μ l	Gradient: H ₂ O (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 μ g/l	[M + NH ₄] ⁺ CsA 1220 > 1203 IS: ASC/CsD	[11–13]
TAC	RTRs (n = 33); RTRs & pancreas (n = 2); and RTRs & HTRs (n = 1) children	10 μ l	Pretreatment with 0.1 mmol zinc sulfate solution and LLE with CAN IV: 10 μ l	Gradient: H ₂ O (A), MeOH (B), both containing 2 mmol ammonium acetate/0.1 FA Online SFE: SecurityGuard C18 cartridge (Phenomenex) Run time: NR LLOQ: 0.5 μ g/l	[M + NH ₄] ⁺ TAC: 821 > 768 IS: ASC	[14]

ACN: Acetonitrile; Ammonium adduct: [M+NH₄]⁺; ASC: Ascomycin; CsA: Cyclosporine A; CsD: Cyclosporine D; FA: Formic acid; HTR: Heart transplant recipient; IV: Injection volume; LgTR: Lung transplant recipient; MeOH: Methanol; R: Recovery; RTR: Renal transplant recipient; TAC: Tacrolimus.

Table 4

Published LC–MS/MS assays for quantification of immunosuppressive drugs in lymphocytes.

Drug	Subjects	Extraction	Chromatographic conditions	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	Gradient: H ₂ O (A), MeOH (B)	LC-MS	[110]
		Lymphocyte isolated from 8 ml blood with BD Vacutainer® CPT™ Cell Preparation Tubes	Online SPE: XTerra™ MS C8	[M + Na] +	[111]
		Cells lysed with MeOH/dry reconstituted with MeOH	Column: XTerra™ MS C18 (Waters)	CsA: 1224.7/NR	[120]
		IV: 40 µl	Run time: 31 min	IS: 27 demethoxy-sirolimus	
		R: 98%	LLOQ: 5 ng/ml; 0.5 fg/PBMC		
CsA	Early RTRs (n = 20); HTRs (n = 10)	Verapamil added prevent efflux of CsA	Gradient: ACN/20 mM ammonium formate buffer pH 3.6 (20:80) (A), ACN/(NH ₄ + COO-) pH 3.6 (80:20) (B)	[M + H] +	[118]
		T-lymphocyte isolated from 7 ml blood with Prepacryte®.	SPE: Water Oasis®, HLB 1 cc, 30 mg	CsA: 1203.7 > 1101.7/1185.7	[121]
		Cells were lysed and protein precipitated with MeOH: ACN: water (1:1:3) followed by SPE	Column: C8, (Thermo Electron Corp)	IS: CsC	
		IV: 100 µl	Run time: 38 min		
		R: CsA and metabolites 73.5–98.6	LLOQ: 0.25 ng/ml		
CsA and metabolites	Early (n = 57) and chronic RTRs (n = 54)	Verapamil added to prevent efflux of CsA	Gradient: 5% ACN (A), 95% ACN (B); both contain 2 mM/l ammonium acetate/0.1 FA	[M + H] +	[122]
		Lymphocyte isolated from 1.5 ml blood with Histopaque 1077 solution	Column: Acquity UPLC RP BEH, C18	CsA: 1202.8 > 156.2.	
		Precipitation solution ACN: MeOH (40:60, v/v); (v:v:v)	Run time: 5 min	IS: CsD	
		IV: NR,	LLOQ: 5 ng/ml		
		R: CsA and metabolites 71.9–78.4%.			
CsA	HTRs (n = 17)	10 ml whole blood incubated with E-Rosette solution, followed by density separation using Histopaque solution.	Isocratic: ACN: 2 mM ammonium acetate: FA (70:30:0.1)	[M + NH] +	[125]
		IV: 1 µl	Column: Supelco LC-CN	CsA: 1,220.8 > 1202.7	
		R: NR	Run time: NA	IS: CsD	

Drug	Subjects	Extraction	Chromatographic conditions	Precursor ion (m/z) > product ion (m/z)	Ref.
EVE	HTRs (n = 36)	LLOQ: 1 pg 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: CAN IV: 20 µl R: 79.4–87.1%.	Gradient: H ₂ O (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 + NH ₄] ⁺ EVE: m/z 975.5 > 908.5 IS: ASC	[132]
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-chlorobutanol. Organic phase dried and reconstituted with MeOH containing 2 mmol/l ammonium acetate/0.1% FA IV: 25 µl R: 74.8–86.7%	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 + NH ₄] ⁺ TAC: m/z 821.6 > 768.5 IS: ASC	[123,124]
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: H ₂ O (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	[119]

Ammonium adduct: [M+NH₄]⁺; ASC: Ascomycin; CsA: Cyclosporine A; CsC: Cyclosporine C; CsD: Cyclosporine; EVE: Everolimus; FA: Formic acid; HTR: Heart transplant recipient; Ion adduct [M + H]⁺; IV: Injection volume; LTR: Liver transplant recipient; LgTR: Lung transplant recipient; MeOH: Methanol; NR: Not reported; PBS: Phosphate buffer solution; R: Recovery; RTR: Renal transplant recipient; TAC: Tacrolimus.

Published LC–MS/MS assays for quantification of immunosuppressive drugs in biopsies from transplanted organs.

Table 5

Drug	Subjects	Extraction/Injection volume/Recovery	Correlation blood vs tissue	Chromatographic conditions	Precursor ion (m/z) > production (m/z)	Ref.
CsA	RTRs (n = 21)	Tissues solubilized in digestion buffer (Proteinase K solution in a tissue lysis (ATL) buffer) at 56°C/2 h. Precipitate with 0.4 M zinc sulfate: MeOH, 20:80 (v:v) IV: 25 μ l R: NR	$r = 0.168$, $p = 0.53$	Gradient: (A): 50% MeOH, MeOH (B), both contain 2 mM ammonium acetate/0.1% FA Online cleaning: POROS R1/20 Column: Luna Phenyl-Hexyl (Phenomenex) Run time: 5.5 min LLOQ: 1 ng/ml	[M + NH ₄] ⁺ CsA: 1220.0 > 1203.0 IS: labeled CsA	[143]
CsA	Biopsies (n = 19) from 7 HTRs	Tissues homogenized with water and mixed with two parts of ACN:MeOH: H ₂ O (1:1:3, v:v:v). Precipitation with ACN. The organic phase was dried and reconstituted with MP's A:B, 65:35 (v:v) IV: 20 μ l 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	[118]
MPA	Biopsies (n = 4) from 4 RTRs	Tissues grounded to a fine powder and reconstituted with PBS (pH 7.4). Add 60 μ l of HCl (0.4 M) and 1 ml of tertiary-butyl methyl ether. Evaporate/reconstitute with 1:1 (v:v) MeOH: H ₂ O. IV: NR R: 97%	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	[145]
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 μ l 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 + NH ₄] ⁺ TAC: 822 > 768 IS: ASC	[123,142]

Drug	Subjects	Extraction/Injection volume/Recovery	Correlation blood vs tissue	Chromatographic conditions	Precursor ion (m/z) > production (m/z)	Ref.
TAC	Biopsies (n = 6) from 2 RTRs	Tissues solubilized in digestion buffer. Mix with 7 μ l of IS + 300 μ l water+1 ml of <i>tert</i> -butyl methyl ether in glass tube. Organic phase evaporated and reconstituted in 50:50 MeOH: H ₂ O IV: 25 μ l R: 70%	NR	Gradient: H ₂ O (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 + NH ₄] ⁺ + TAC: 821.5 > 768.6 IS: ASC	[144]

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