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PHARMACOKINETICS AND DISPOSITION

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

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Abstract

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

Method The liquid chromatography coupled to mass spectrometry (LC-MS) method was developed and validated for determining mycophenolic acid (MPA) and its glucuronide metabolite (MPAG) in oral fluid and plasma, with both matrices presenting a detection limit of 1 ng/mL for MPA and 5 ng/mL for MPAG. Both analytes were analysed after a simple protein precipitation procedure. Transplanted-kidney samples of oral fluid and blood were collected from 13 patients that were hospitalised and kept at − 80 °C until analyses.

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

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

Keywords LC-MS · Oral fluid · Plasma · Mycophenolic acid · Mycophenolic acid glucuronide · Pharmacokinetic

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Introduction

According to the latest United Nations reports, the world's population is ageing and with that chronic diseases are on the increase, including kidney failure [1–3]. The first choice of treatment is kidney transplantation, and in 2017 more than 5000 transplants were performed in Brazil [1, 2, 4]. In kidney transplant therapeutic regimens, one of the most used immunosuppressive drugs to prevent allograft rejection is mycophenolic acid (MPA) in association with corticosteroids and calcineurin inhibitors [5–7]. MPA, in its active form, can be used in two ways to increase absorption: as the pro-drug mycophenolate mofetil (MMF) or as mycophenolate sodium (EC-MPS) [8–10]. Metabolization of MPA after absorption is carried out by UDP-glucuronosyltransferases (UGTs) into a

pharmacologically inactive MPA-7-*O*-glucuronide (MPAG), the main metabolite, and its minor active metabolite MPA acyl glucuronide (AcMPAG) [8, 10]. The mechanism of action of MPA is the selective and reversible inhibition of the rate-limiting enzyme inosine monophosphate dehydrogenase type 2 (IMPDH-2) in de novo purine biosynthesis of guanine nucleotides in lymphocytes [6, 11, 12].

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

Methods to detect MPA and its glucuronide metabolite in a different biological matrix have already been described, but there has only been one study using oral fluid to detect MPAG [8, 13, 15, 17–20]. This study aimed to develop a sensitive LC-MS method with a simple and rapid sample clean up to quantify MPA and MPAG in oral fluid and plasma in order to apply it in samples from kidney transplant patients using a limited sampling strategy and try to establish a correlation between them.

Material and methods

Chemicals and reagents

Standards of mycophenolic acid (MPA) and ketoprofen, used as an internal standard (IS), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mycophenolic acid glucuronide (MPAG) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The acetonitrile, methanol and formic acid were all HPLC grade from Merck (Frankfurt, Germany). Zinc sulphate was reagent grade. Ultrapure water was obtained using a Milli-Q Plus system (Millipore, Bedford, MA, USA). Drug-free plasma and oral fluid were donated by the volunteers.

Apparatus

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

Patient population and ethics

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

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

Study design

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

Liquid chromatography-mass spectrometry

Chromatographic separation was performed using an Agilent Zorbax Eclipse Plus C18 column (4.6 × 150 mm, 3.5 μm particle size) (Torrance, CA, USA). Gradient condition was used, consisting of solvent A (H_2O + 0.1% formic acid), solvent B (acetonitrile +0.1% formic acid) and solvent C (methanol +0.05% formic acid) as follows: A → 57% B → 23% C → 20% (0–5.0 min), A → 50% B → 42% C → 8% (5.0–13 min), A → 57% B → 25% C → 18% (13.0–16.0 min). The flow rate was set at 0.7 mL/min and the temperature maintained at 35 °C.

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

Sample preparation and cleaning procedure

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

The plasma (50 μL) was pipetted and transferred into 1.5 mL a polypropylene conical tube, 25 μL of mix solution with MPA and MPAG was added and then vortex mixed for 10 s. The IS (25 μL) and 250 μL of an ice-cold precipitating solution (0.1%) formic acid in methanol

and 0.1% zinc sulphate in water, (70:30) were added and the vortex mixed for 20 s.

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

Standards solutions, analytical curves and quality controls

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

Bioanalytical method validation

FDA bioanalytical guidance complemented by the EMA was used to perform the method validation [21, 22]. Selectivity, to assure the absence of interfering peaks in the quantification of MPA and MPAG, was performed by analysing blank samples from six different sources and a pool of 20 donors, and spiked with acetaminophen, prednisone, diclofenac, dexamethasone, ciprofloxacin and cyclosporine drugs that are commonly used by kidney transplant patients. For plasma, an additional assay was performed using lipemic and hemolyzed blood samples. The lowest concentration giving a response of at least three times the average baseline noise ($S/N > 3$) was defined as the LOQ and the lowest concentration that could be measured with CV with a bias lower that 20% was defined as LLOQ.

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

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

The stability of the analytes in the oral fluid and plasma was evaluated following the guidelines [21, 22] (see supplementary material).

Pharmacokinetic analysis

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

Results and discussion

Bioanalytical method validation

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

Analysis of the seven-point calibration curve (5–500 ng/mL for MPA and 10–500 ng/mL for MPAG) covering the expected range demonstrates adequate correlation coefficients (r), showing that the method was linear for both analytes (Table 1). Only one study has described the presence of MPAG in oral fluid with a concentration lower than 10 ng/mL and it used an LC-MS/MS method [13, 15, 23].

Accuracy was accessed by calculating the bias (Table 1). For all the QC samples, run precision (CV%) was calculated and presented adequate results, as recommended by the guidelines (Table 1). Extraction recovery was around 85 ± 5% of the standard concentration for MPA and MPAG. Once patient samples could present MPA and MPAG concentrations up to 500 ng/mL, dilution integrity was performed and showed adequate results for accuracy and precision.

Stability tests for LQC, MQC, and HQC in both matrices were performed. The samples were stable in plasma and oral fluid at room temperature (20 °C ± 2) for 8 h and post-extraction in the auto sampler for 12 h. After 3 months at − 20 °C (short-term stability) and after three freeze-thaw cycles, the MPA showed degradation in both matrices; however, MPAG was shown to be stable in these conditions. After 6 months (long-term stability) at − 20 °C, both analytes were not to be stable in plasma and oral fluid (supplementary material). Short-term stability at − 80 °C demonstrates that MPA and MPAG were stabled under this condition. Besides that, it has already been described in the literature that samples are

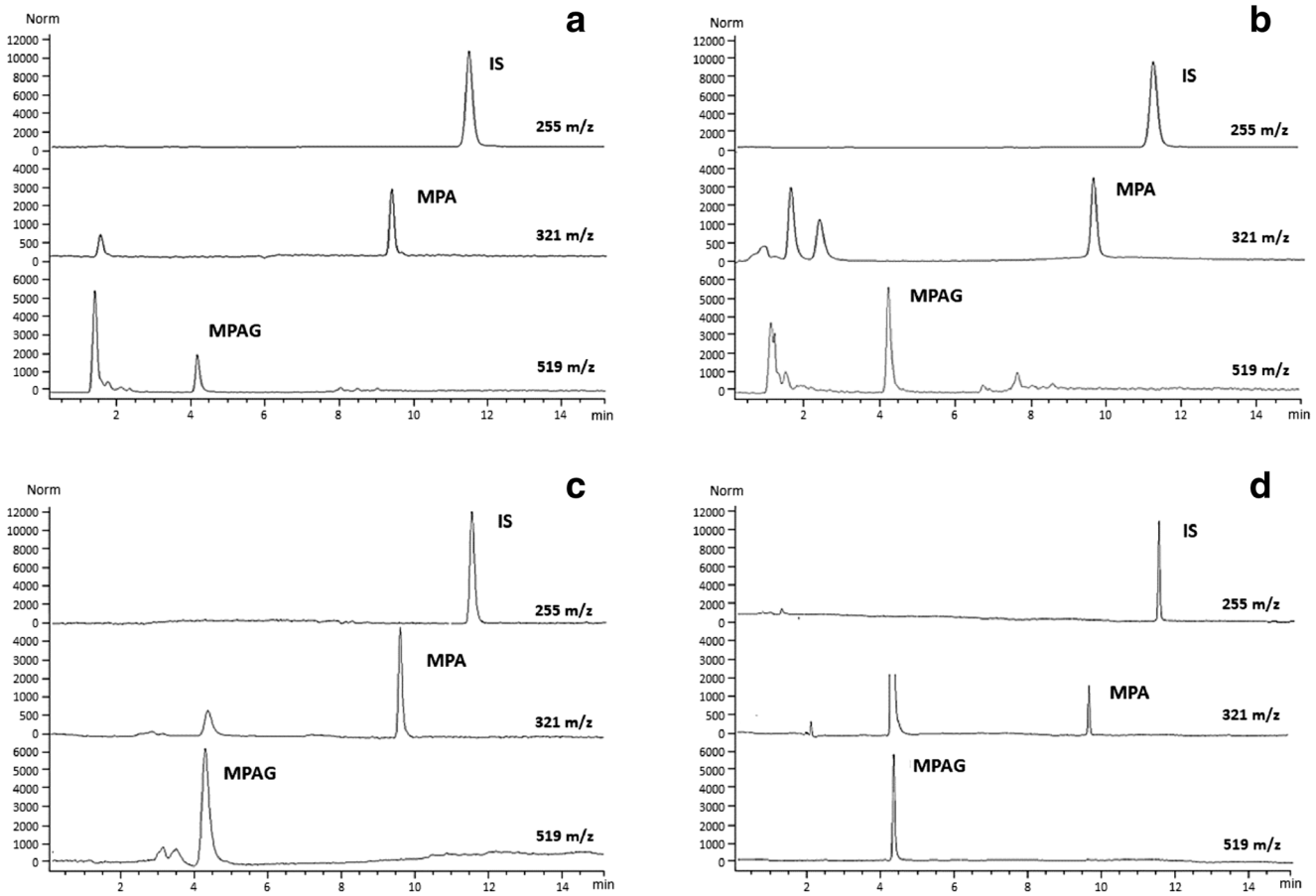

Fig. 1 Representative LC-MS chromatogram (SIM mode) of the analytes MPA, MPAG and IS. **a** Oral fluid, **b** plasma, both spiked with MPA and MPAG standards, at 100 ng/mL, and interfering drugs. **c** Sample of oral fluid and **d** sample of plasma, both from kidney-transplanted patient

stable in plasma at − 80 °C for at least 6 months, so kidney-transplanted samples were kept at this condition until analysis [10, 13, 19, 24].

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

Matrix effect

The post-extraction addition approach was used to evaluate matrix interferences [21, 22]. No matrix effect was observed in both biological fluids since the CVs for all analytes were lower than 15%, as recommended by the

Table 1 Limit of detection (LOD), limit of quantification (LOQ), correlation coefficient (*r*), accuracy and precision (between-run) of MPA and MPAG in oral fluid and plasma*

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

[a] Concentration (ng/mL)

[b] Mean data of 3 days of analysis

*Analysis performed in three concentration levels: lower quality control (LQC), middle quality control (MQC) and higher quality control (HQC)

guidelines. Carry over was not observed in the corresponding chromatogram, after injecting the highest concentration of the analytical curve.

Pharmacokinetics analysis

In this study, to determinate plasma and oral fluid concentrations of MPA and MPAG, the validated LC-MS method was applied to samples from 13 kidney transplant patients. To analyse the results, a non-compartmental model was used as it has already been described for MPA and MPAG in several studies [17, 19, 25–27]. The plasma and oral fluid concentrations of MPA and MPAG were found to be variable for both analytes (Fig. 2).

The median MPAG $AUC_{0–12}$ was about 10-fold higher than the MPA $AUC_{0–12}$ values (Table 2). These higher MPAG values were expected due to fast MPA metabolization in MPAG and AcMPAG [7, 25, 28–30]. Oral fluid presented, for both analytes, a lower concentration and $AUC_{0–12}$ than in plasma, which can be explained by the fact that in the plasma the total fraction was analysed and in the oral fluid only the free fraction was assessed, that is, ± 3% for MPA and ± 15% for MPAG [7, 31]. The target MPA $AUC_{0–12}$ value in blood, recommended for sufficient immunosuppression in kidney transplant recipients receiving MMF, is 30–60 μg h/mL (40–60 μg h/mL when EC-MPS is used) and the therapeutic range was reached in all the patients included in our study, with a mean of 55.83 μg h/mL [32]. Comparing the $AUC_{0–12}$ profiles in plasma and oral fluid, these were similar for MPA and MPAG, as shown in Fig. 2.

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

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

One of the biggest advantages of using oral fluid is that collection is non-invasive and it is gaining considerable importance in drug monitoring [15, 33]. Collection of oral fluid does not require trained personnel, it can be used in adults and children as it is less stressful, and it can be carried out anywhere. This matrix has great potential for drug monitoring but it has some characteristics that should be considered, such as salivary pH, molecular weight, and percentage of the free fraction. The use of other drugs at the same time can also change saliva pH. Other problems with oral fluid are that it can suffer contamination from residual food particles and blood, as well as the low production of it, which can influence viscosity and make it difficult to collect and process samples. In our

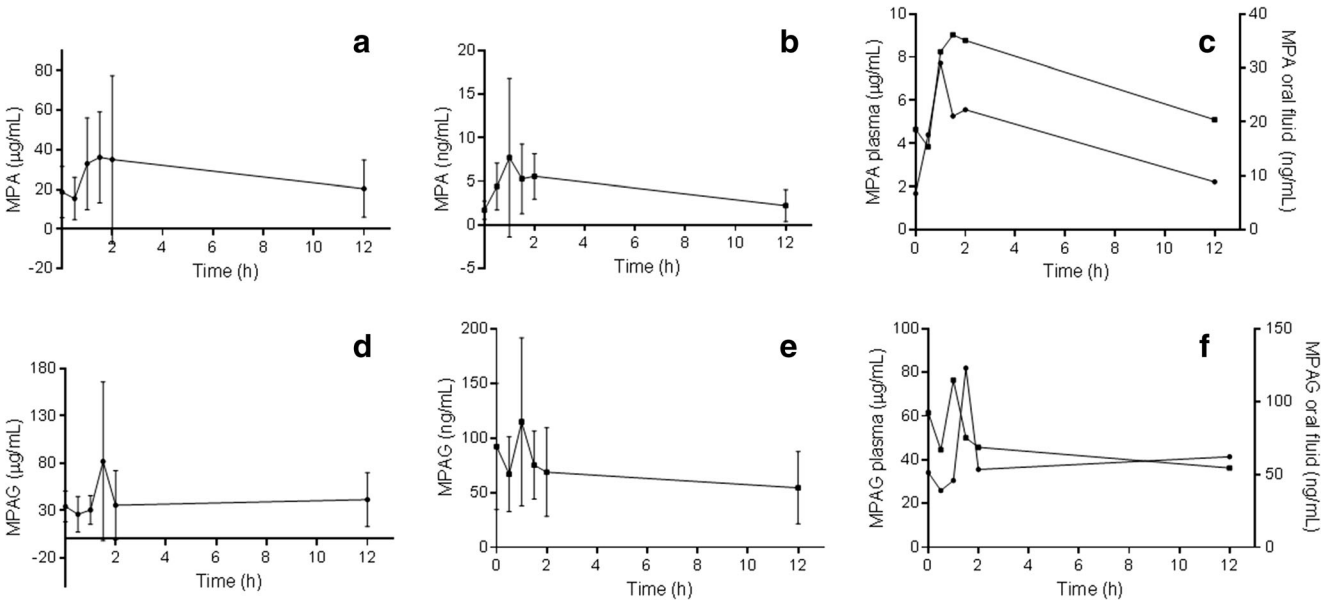

Fig. 2 Average concentration-time profile for MPA and MPAG (**a**) MPA in plasma, (**b**) MPA in oral fluid, (**c**) Comparison of MPA in oral fluid and plasma plasma , oral fluid (**d**) MPAG in plasma, (**e**) MPAG in oral fluid, and (**f**) Comparison of MPAG in oral fluid and plasma plasma oral fluid

Table 2 The main pharmacokinetics parameters of MPA and MPAG after an oral administration of 750 mg of MMF or EC-MPS to 13 renal transplant patients

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

*Mmean (SD)

study, we observed low oral fluid production in some patients, and we had to ask them to keep the Salivette® on their mouths longer. We did not use anything to promote salivation because this can influence the pH of the oral fluid and we were afraid it could change the plasma/oral fluid correlation [34].

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

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

Despite scarce information available in the literature concerning MPA pharmacokinetics in plasma and its relationship with an oral fluid, our results are in accordance with those previously reported (supplementary material) [6, 25, 29, 30, 35–37].

Conclusions

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

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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