Analysis of Mycophenolic Acid in Saliva Using Liquid Chromatography Tandem Mass Spectrometry

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Abstract: Salivary levels of the immunosuppressive agent, mycophenolic acid (MPA), may provide a convenient and noninvasive method for drug monitoring. An analytical method was developed and validated for quantification of salivary MPA using liquid chromatography tandem mass spectrometry. Sample preparation included addition of 50 μL internal standard solution [500 μg/L indomethacin in methanol] to 100 μL saliva sample, followed by protein precipitation with 200 μL acetonitrile. Supernatants were dried and reconstituted in 100 μL of 85:15% (vol/vol) mixture of methanol and water containing 0.05% formic acid and 20 μL was injected onto the analytical column. The mobile phase comprised a gradient mixture of methanol and 0.05% formic acid, giving a total run time of 7.5 minutes. Chromatograms were obtained using mass transitions of m/z 319.0 → 190.8 for MPA and m/z 355.9 → 312.2 for indomethacin. The calibration curve was linear over a concentration range of 2.5 to 800 μg/L (r = 0.9999) and the recovery of MPA from saliva was > 90%. The inaccuracy was < 10% and intra- and interday coefficient of variation ranged from 2.8% to 5.2%. Mean ± SD of MPA concentrations in saliva (n = 100) obtained from 11 kidney transplant recipients was 31.4 ± 32.3 μg/L (range: 2.6 to 220.4 μg/L) and correlated well with total (r = 0.909) and unbound (r = 0.910) MPA concentrations in plasma. In conclusion, a simple, sensitive, and specific method was developed and validated for quantification of MPA in saliva. Additional clinical studies are required to establish the usefulness of this specimen in the clinical management of organ transplant recipients.

Key Words: mycophenolic acid, saliva, concentration, LC-MS/MS, transferrin

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Mycophenolic acid (MPA) is used as an immunosuppressant in combination with a calcineurin inhibitor and a corticosteroid for the prevention and treatment of allograft rejection.[1] In vivo it reduces guanine nucleotide biosynthesis by inhibiting inosine 5'-monophosphate dehydrogenase.[1] MPA exhibits variable pharmacokinetic characteristics; therefore, as a guide to dose individualization, monitoring MPA concentrations may improve posttransplant outcomes.[1–3] Currently, 2 large randomized, multicenter, prospective clinical trials are under way in the United States and Europe that are aimed to address the importance of therapeutic drug monitoring for MPA as compared to the fixed-dose regimen.[4]

In plasma, MPA is highly bound to serum albumin[5] with an average free fraction of approximately 2% to 3%.[6] Because unbound or free concentration represents the pharmacologically active form of a drug, monitoring unbound MPA may prove beneficial in clinical practice. It has been shown that the unbound concentration of MPA may associate to a greater extent than the total concentration with therapeutic outcomes.[5,7,8] In addition, there is considerable intra- and intersubject variability in the degree of binding of MPA to plasma proteins.[7,9,10] The unbound fraction of MPA is shown to be dependent on the concentration of serum albumin and other factors, that is, renal function, the concentration of mycophenolic acid-glucuronide (MPAG) and coadministration of furosemide.[5]

Several methods have been used to quantify unbound MPA in plasma including ultrafiltration followed by chromatographic analysis of MPA[11–13] and equilibrium dialysis using radiolabeled MPA[5]; however, these methods are laborious and require approximately 1 mL plasma. Saliva offers a noninvasive specimen for drug analysis and may prove useful for routine therapeutic monitoring of immunosuppressants including cyclosporine[14] or MPA. Saliva represents a natural ultrafiltrate of plasma; therefore salivary concentrations of drugs, in theory, should represent the unbound concentration.[15] An unstressful sampling versus venipuncture is another advantage of saliva, thereby allowing repeated sampling in a nonmedical environment for adult and pediatric patients alike.

In this report, we present the development of a simple and sensitive assay for quantification of MPA in saliva using liquid chromatography tandem mass spectrometry (LC-MS/MS), assay validation process according to the guidelines set by the Food and Drug Administration

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(FDA) of United States[16] and some preliminary data on the correlation between salivary MPA concentrations with total and unbound MPA levels in stable kidney transplant recipients.

MATERIALS AND METHODS

Chemicals

Standard samples of MPA (98.9% pure), MPAG (98.7% pure), and acyl-mycophenolic acid-glucuronide (AcMPAG, 97.7% pure) were kindly donated by Roche Laboratories (Palo Alto, CA). A 500 μg/L solution of indomethacin (INDO, Alfa Aesar, Ward Hill, MA) in methanol served as internal standard for the assay. All reagents and solvents were high-performance liquid chromatography grade. Substocks of MPA in methanol (1, 5, and 50 mg/L) were prepared and used to spike saliva.

Calibration Curves

Calibrator and quality control (QC) standards were prepared using pooled unstimulated whole saliva collected from at least 6 healthy volunteers (IRB Approval#HU0203-120). For each batch analyzed, a 7-point calibration curve (2.5, 25, 50, 100, 300, 500, 800 μg/L) of MPA in saliva was constructed using $1/x^2$ linear regression, and in-house QCs at 3 concentrations (10, 200, and 600 μg/L) corresponding to low, medium, and high levels. Five hundred microliters of all calibrators and QCs were aliquoted into 2 mL cryovials and maintained at −20°C until use.

Sample Extraction

Extraction of MPA from saliva was carried out by protein precipitation. Calibrators, QCs, or patient samples were thawed in a shaking water bath at 37°C for 5 minutes. The samples were then sonicated for 10 seconds using a Branson handheld Sonifier (Danbury, CT) and 100 μL were pipetted into a microcentrifuge tube, followed by the subsequent additions of 50 μL methanol containing INDO (500 μg/L) and 200 μL acetonitrile. The tubes were vortex mixed for 90 seconds and centrifuged at 16,000*g* for 5 minutes. The supernatants were carefully aspirated into glass culture tubes and dried at 50°C in a centrifugal evaporator (Thermosavant, Holbrook, NY) after which they were reconstituted with 100 μL of 85:15% (vol/vol) of methanol and 0.05% formic acid in deionized water and a 20 μL aliquot was injected onto the analytical HPLC column.

LC-MS/MS Conditions

Liquid chromatography was performed on a Perkin Elmer series 200 HPLC system consisting of an autosampler and 2 micropumps (Norwalk, CT). All mass spectrometry conditions were already described in an earlier publication from our group[11] using an API 2000 Mass Spectrometer (Sciex, Toronto, Canada). The analytes were detected in the negative ion mode using the mass transitions of m/z 319.0→190.8 for MPA, m/z 355.9→312.2 for INDO, and m/z 495.0→319.2 for both MPAG and AcMPAG. The analytical column was Zorbax Rx C_8 (150 mm × 4.6 mm, 5 μm) from Agilent Technologies (Palo Alto, CA) and mobile phase was a gradient mixture of methanol and deionized water containing 0.05% formic acid. Elution of MPAG, AcMPAG, MPA, and INDO from the chromatographic column was carried out with a gradient mobile phase composition consisting of 72:28% (vol/vol) methanol: 0.05% formic acid for the first 3.5 minutes at a flow rate of 0.6 mL/min then switching to 85:15% (vol/vol) methanol: 0.05% formic acid at a flow rate of 0.7 mL/min for the next 2.5 minutes and switching back to 72:28% (vol/vol) methanol: 0.05% formic acid composition for 1 minute with a flow rate of 0.7 mL/min.

Because of the potential problems with insource fragmentation of glucuronide metabolites to MPA, it was necessary to separate traces of MPA, MPAG, and AcMPAG chromatographically. Additionally, ion-suppression test was performed to evaluate the effect of salivary proteins on the ionization of MPA and INDO.[17] For this, a combined mixture of the analytes (1 mg/L each) in mobile phase was infused continuously onto the mass spectrometer and the residues extracted from blank saliva were injected simultaneously via a 3 way T-valve.

Assay Validation

The lower limit of quantification and limit of detection were defined at a signal to noise ratio of 5:1 and 3:1, respectively. The recovery of the extraction procedure was assessed by comparing the peak areas obtained from an extracted saliva-based standard of MPA or INDO with the peak areas of these analytes in methanol. To evaluate intraday coefficient of variation of the assay, QCs were analyzed 6 times on the same day. Interday coefficient of variation and accuracy was evaluated by measuring the QC concentrations over 10 days using a separate calibration curve for each set.

Stability studies were carried out at 10 and 600 μg/L MPA in triplicate. For short term stability study, samples were kept on the bench top for 5 hours at room temperature and for freeze-thaw stability samples were subjected to 3 cycles of freezing at −20°C and thawing unassisted at room temperature. To evaluate autosampler stability, dried and reconstituted extracts were kept in the autosampler for 14 hours and then analyzed. To determine stock solution stability, methanolic-based stock solutions of MPA and INDO were kept at room temperature for 8 hours and the analyte loss was compared against freshly prepared samples.

Analysis of Patient Samples

Upon obtaining IRB approval (IRB#0159-03 and 0174-04) and informed consent, parallel blood and saliva samples were collected immediately before the morning dose and at 1, 2, 3, 4, 5, 7, 9, 10, and 12 hours post-MPA dose from 11 kidney transplant recipients attending transplant services clinics at Rhode Island Hospital (Providence, RI). All patients were receiving 500 to 1000 mg, twice daily dose of mycophenolate mofetil

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(Cellcept®, Roche Laboratories). Unstimulated saliva samples were collected by passive drool, without stimulation, into a plastic cup within a 5 minute period of blood collection and stored at −80°C until analysis. The patients remained fasted for the first 2 hours of sampling but then were allowed standard hospital meals. Total and unbound concentrations of MPA were measured using HPLC-UV[18] and ultrafiltration followed by LC-MS/MS,[11] respectively. Data analysis was performed using SPSS version 11.0 software (SPSS Inc, Chicago, IL). Pearson Correlation coefficients were reported for association between the 2 variables and differences between 2 groups were determined using t test.

Analysis of Transferrin in Saliva

We have investigated the possibility of blood leakage into saliva by measuring salivary concentration of transferrin using a commercially available kit from Salimetrics LLC (State College, PA). This salivary blood contamination enzyme immunoassay kit measures transferrin, a large protein (molecular weight 76,000) that is present in abundance in blood but normally is present in trace amounts in saliva. The manufacturer of this technique recommends that values > 1 mg/dL salivary transferrin should be considered as candidate for exclusion for other salivary tests.

RESULTS

The elution times for MPA and INDO were approximately 5 and 6.5 minutes respectively. The HPLC method resolved the MPA peaks from MPAG and AcMPAG, therefore eliminated the possibility of interference due to in source fragmentation of these metabolites.[11] A typical chromatogram of MPA extracted from saliva obtained from a kidney transplant recipient is shown in Figure 1, indicating MPA peak was well separated from MPAG peak. Some degree of insource fragmentation of MPAG to MPA was observed; hence, the chromatogram shows traces of MPA at the MPAG retention time. However, no AcMPAG peak was observed at 3.8 minutes, the expected retention time for AcMPAG, in any of the patient saliva samples analyzed. Ion-suppression studies revealed that the time of matrix or water dips did not interfere with the elution times of MPA and INDO (Fig. 2).

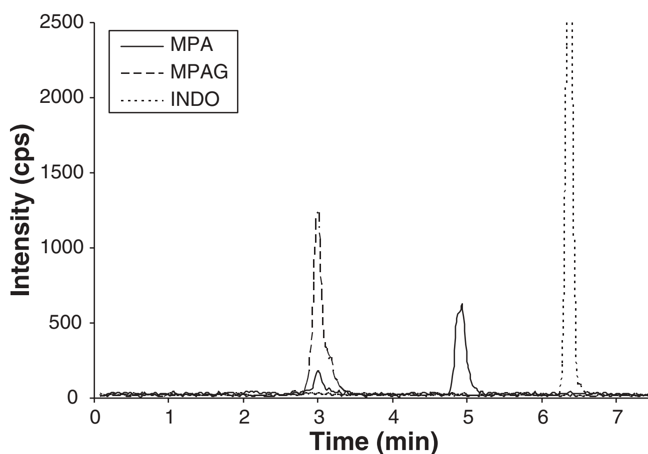

FIGURE 1. Chromatogram of MPA, MPAG, and internal standard indomethacin extracted from saliva sample from a representative kidney transplant recipient. The analytes were detected in the negative ion mode using the mass transitions of m/z 319.0→190.8 for MPA, m/z 495.0→319.2 for MPAG and m/z 355.9→312.2 for indomethacin.

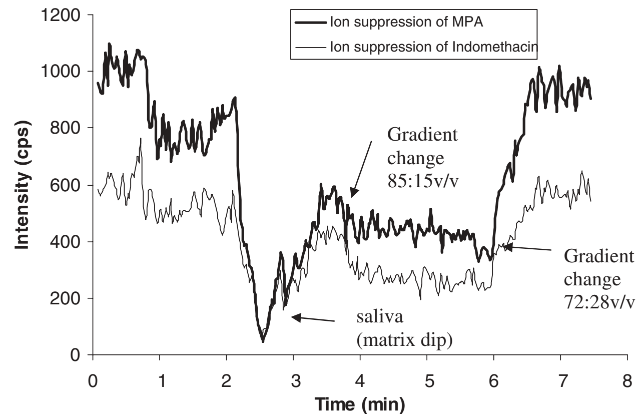

FIGURE 2. Effect of saliva extract on the suppression of ionization of MPA and indomethacin indicates that matrix dip occurs at time different from retention times of MPA or indomethacin.

The validation data represent results of 10 separate sets of calibration and QC standards. The lower limit of quantification was 2.5 μg/L and limit of detection was 1 μg/L. The assay was linear over a working range of 2.5 to 800 μg/L for MPA (Fig. 3) (correlation coefficient = 0.9999). The performance of the assay (Table 1) fulfilled the overall FDA validation criteria. The recovery of MPA and INDO from saliva samples was > 90%. The results of the stability studies indicate that MPA is stable in saliva-based standards under the experimental

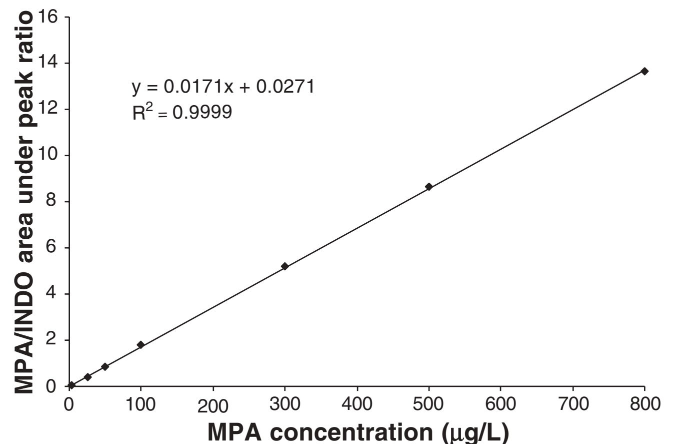

FIGURE 3. Saliva-based calibration curve for MPA.

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TABLE 1. Results of MPA Recovery, Accuracy, Intra- and Inter-day Precision and Stability Studies for MPA Using Saliva-Based Quality Control Standards

Concentration (μg/L)	Recovery (n = 6)	Accuracy% (n = 10)	Intraday CV% (n = 6)	Interday CV% (n = 10)	Stability Studies: Freeze Thaw (n = 3)	Stability Studies: Short Term (n = 3)	Stability Studies: Autosampler (n = 3)
10	91.3 ± 4.7	99.8 ± 5.2	2.8	5.2	103.6 ± 7.1	106.0 ± 3.6	95.0 ± 6.6
200	92.7 ± 3.0	99.8 ± 7.7	3.4	4.1	NA	NA	NA
600	94.8 ± 1.3	99.4 ± 6.9	3.4	3.6	98.9 ± 1.1	98.7 ± 2.7	98.2 ± 0.6

All plus–minus data are mean ± SD.
CV%, coefficient of variation; NA, not available.

conditions described above (Table 1). The loss of analytes at room temperature from methanolic stock solutions of MPA and INDO was 0.6% and 10%, respectively.

All of 11 patients studied were male, Caucasian and on immunosuppressive therapy with tacrolimus and prednisone. The mean ± SD of patient age was 55 ± 7 years, and time postkidney transplantation was 1.78 ± 1.74 years. Figure 4 depicts the average MPA concentrations over a 12-hour dosing interval in saliva and its total and unbound concentrations in plasma. Mean ± SD of saliva concentration was 31.4 ± 32.3 μg/L (range: 2.6 to 220.4 μg/L, $n = 100$). Salivary concentration of MPA before administration of Cellcept morning dose was remarkably higher than saliva concentrations at other times with a considerable variability (79.8 ± 63.7 μg/L). With the exception of morning trough, the average salivary concentration of MPA was well correlated with its total ($r = 0.909$, $P = 0.001$) or unbound concentration ($r = 0.910$, $P = 0.001$) at other times. The concentration of saliva transferrin was 1.14 ± 0.89 mg/dL ($n = 11$) in samples obtained at morning trough as compared to 0.38 ± 0.24 ($n = 89$) at other times during the 12-hour dosing interval

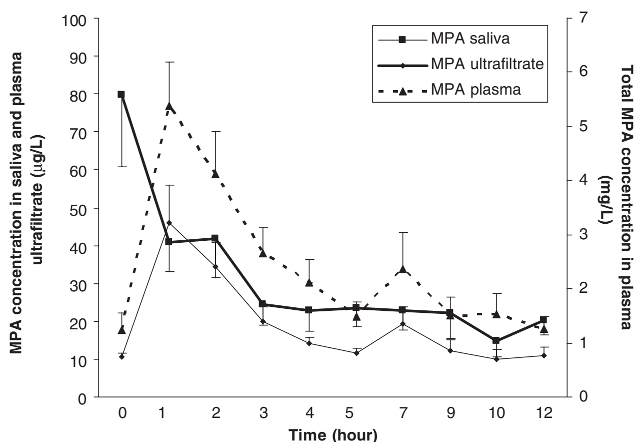

FIGURE 4. Average concentration–time profile for MPA concentrations in saliva as compared with plasma (total concentration) and plasma ultrafiltrate (unbound concentration) from 11 stable kidney transplant recipients (error bars represent standard error of the mean).

($P = 0.007$). It seems that MPA concentration in saliva is elevated in fasting patients in the morning possibly because of the presence of blood in saliva.

DISCUSSION

The LC-MS/MS method described in this manuscript is a highly reliable, simple, and sensitive assay requiring a small volume of saliva. Initially we have tried a previously reported[11] solid phase extraction procedure for the extraction of MPA from saliva, but have experienced poor and nonreproducible recovery. Our aim was to eliminate the need for a lengthy extraction process and we have succeeded in establishing a simple yet reproducible protein precipitation process rendering consistent and high recoveries for both MPA and INDO. We have also found that it is essential to break salivary protein aggregates by sonication of saliva samples before extraction. The assay was sensitive in quantifying MPA concentrations in saliva during a 12-hour dosing interval and have met FDA guidelines at all levels.

Because of its noninvasive collection method, saliva monitoring of drugs and hormones have gained considerable importance.[19–21] The collection method is less stressful for adults and children and can be conducted in the convenience of ones home, without the need for trained personnel. Furthermore, multiple saliva samples can be obtained at regular intervals to allow estimation of abbreviated or full area under the concentration-time curves. The distribution of drugs into saliva is dependent on factors such as degree of plasma protein binding, molecular weight, lipid solubility, ionization, and salivary pH.[15,20] The degree of ionization of a substance would determine if saliva to plasma ratio remains unaffected by saliva pH, for instance, saliva to plasma ratio of neutral drugs or those with pK_a below 5.5 or above 8.5 should not be affected by salivary pH variation.[22] The pK_a value for MPA is 4.5[23] so we can predict changes in salivary pH would not influence its saliva to plasma concentration ratio.

The disadvantages of salivary drug monitoring are possible contamination, with food particles and blood, and difficulty in pipetting due to the viscosity of saliva.[24] The contamination problem may be alleviated by asking the donor to rinse their mouth before saliva collection and the viscosity problem resolved by using a Sonifier to

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break up salivary mucin. We have observed exceptionally high morning trough concentrations of MPA in the saliva obtained from 9 of the 11 patients when compared with the remaining time points. All patients in this study were asked to report to the hospital, fasted, early in the morning (7:00 AM). It is possible that teeth brushing and flossing may lead to some degree of bleeding and contamination of saliva with blood possibly resulting in high transferring and MPA concentrations at this point in time. In this study we have not assessed the possibility of alleviating this problem by rinsing the mouth, consumption of food and drink or stimulation of saliva production; however, the concentration of transferrin decreased in all patients after they started to eat and drink. Further studies are required to develop an optimal sampling process and to investigate the effects of fasted versus fed condition and also saliva stimulation on the salivary concentrations of MPA.

CONCLUSIONS

In conclusion, we have developed a simple, sensitive, and reproducible method for determination of MPA in saliva and have validated this assay according to FDA guidelines.[16] Our results show a good correlation between saliva concentration with total and unbound concentrations. Saliva concentrations of MPA were higher at morning trough most probably because of blood contamination at this point in time. Further clinical studies are required to establish the best possible sample collection method and to study the clinical utility of this assay in optimizing immunosuppressive therapy in recipients of organ transplantation.

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