Determination of Cyclosporine in Saliva using Liquid Chromatography–Tandem Mass Spectrometry

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Abstract: Saliva may offer an alternative specimen for the therapeutic monitoring of cyclosporine (CsA) in children and patients with difficult venous access. For a highly protein-bound drug such as CsA, saliva may also provide a practical approach for measuring the unbound concentration. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is ideally suited for the measurement of drugs in saliva. A solid-phase extraction technique, analytic liquid chromatography over an Aqua Perfect C_{18} column, maintained at 65°C, and electrospray tandem mass spectrometry were used to quantify CsA in saliva. The method used cyclosporine C (CsC) as the internal standard. Mobile phase comprised of a 97:3 vol/vol mixture of methanol and 30 mmol ammonium acetate at a flow rate of 0.5 mL/min. Chromatograms using mass transitions of *m/z* 1219.9 → *m/z* 1202.9 for CsA and *m/z* 1235.9 → *m/z* 1218.9 for CsC were obtained. The calibration curve was linear from 1 to 300 μg/L with correlation coefficient values ranging from 0.9732 to 0.9968). The lower limit of quantification was 1 μg/L, and limit of detection was 0.6 μg/L with an average extraction recovery of 84.7 ± 2.6% for CsA and 93.7 ± 4.4% for CsC from the saliva matrix. The accuracy of the method ranged from 92% to 104.7%, and the intra- and interrun coefficients of variation were 6.9–12.2% and 8.3–12.1%, respectively. The correlation coefficient value between the CsA concentration measurements in 15 paired blood–saliva samples from kidney transplant recipients was 0.695 ($P = 0.006$). The noninvasive and simple method of saliva collection coupled with the LC-MS/MS quantification technique for CsA analysis would generate novel data that could benefit patients undergoing CsA therapy.

Key Words: cyclosporine, saliva, concentration, liquid chromatography tandem mass spectrometry

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Cyclosporine (CsA) is a potent and effective immunosuppressive agent widely used following organ transplantation[1] or for the treatment of autoimmune diseases.[2] Upon complex formation with cyclophilin, CsA inhibits the activation of calcineurin, a calcium/calmodulin-related phosphatase, thus preventing the translocation of a transcription factor; nuclear factor of activated T cells (NF-AT). Cyclosporine also inhibits activation of another transcription factor, NF-κB, resulting in suppression of T-cell activation by the means of inhibiting interleukin-2 gene expression.[3]

Because of the narrow therapeutic index and significant intra- and interindividual variability in the pharmacokinetic characteristics of CsA, the therapeutic concentrations of this drug must be routinely monitored.[4] However, despite routine monitoring, many patients experience organ rejection[5] and/or adverse effects including nephrotoxicity,[5] hepatotoxicity,[6] neurotoxicity,[7] and gingival hyperplasia.[8]

Cyclosporine is more than 99% bound to blood cells and plasma proteins,[9] leaving a very small fraction of unbound (free) drug. It is generally believed that the pharmacological action of a highly protein-bound drug is dependent on the drug concentration at the receptor site, which is directly related to the concentration of the unbound drug in plasma.[10] In addition, it has previously been shown that the unbound CsA concentration in plasma was associated to a greater extent than the total concentration with the incidence and severity of heart transplant rejection.[11] Determining CsA unbound concentration may therefore prove beneficial in the management of CsA therapy.

Several methods including ultracentrifugation,[12] erythrocyte partitioning,[13] equilibrium dialysis using stainless steel chambers,[14] and microdialysis[15] have been used to separate and measure the unbound concentration of CsA in plasma. However, because of the hydrophobic nature of the CsA molecule, many of these methods, with the exception of equilibrium dialysis, provide unreliable estimates for the CsA unbound concentration.[9] Furthermore, the equilibrium dialysis technique using radiolabeled CsA is a complex and laborious method that is not suitable for measurement of CsA unbound concentration on a routine basis.

In the absence of active transport mechanisms, only the unbound portion of a drug is capable of diffusing across the

membrane of salivary gland capillaries into saliva. Hence, the measured drug concentration in saliva should represent the unbound drug concentration.[16] Cyclosporine is a neutral lipophilic molecule that is expected to diffuse into saliva *via* passive diffusion.[17] Consequently, the total concentration of CsA in saliva should correlate well with the plasma or blood unbound concentration, making CsA an ideal candidate for salivary therapeutic drug monitoring (STDM).[18] The objective of this study was to develop a sensitive, precise, and robust method for quantifying the CsA concentration in saliva using liquid chromatography–tandem mass spectrometry (LC-MS/MS) and to validate this method according to the guidelines for analytic method development provided by the Food and Drug Administration of the United States (FDA).[19]

MATERIALS AND METHODS

Saliva and Blood Samples

For calibration purposes unstimulated drug-free saliva and EDTA whole blood samples were obtained from 10 and 2 healthy volunteers, respectively [University of Rhode Island Institutional Review Board (IRB) Approval No. HU0203-120]. In addition, subsequent to signing of an informed consent form, blood and saliva samples were obtained from 15 kidney transplant recipients attending the outpatient clinic at the Rhode Island Hospital, Providence, Rhode Island [IRB No. 0222-02]. The unstimulated saliva was collected by asking patients to accumulate their saliva over 4–5 minutes and spit it into silanized plastic cups. To obtain a wide range of CsA concentrations in patient samples, no restriction on the time post CsA dose was implemented.

Chemicals

Samples of CsA, cyclosporine C (CsC), and cyclosporine D (CsD) were generously donated by Novartis Pharma AG (Basel, Switzerland). Ammonium acetate was obtained from Acros (Morris Plains, NJ), and HPLC grade acetonitrile, heptane, isopropanol, and methanol were purchased from Fisher Scientific (Fair Lawn, NJ).

Calibration Curves

A series of saliva-based calibrators (1, 10, 50, 100, 150, and 300 μg/L) and quality control standards (2, 75, and 200 μg/L) were prepared. All calibrators and quality control solutions were maintained at −20°C before use. A working internal standard/precipitating solution of 0.02 μg/L CsC in 94:6 vol/vol acetonitrile:water was prepared from the stock solution of CsC (1 g/L) in methanol. For the measurement of CsA in whole blood, calibrators containing 5, 25, 50, 100, 250, 500, 1000, and 2500 μg/L of CsA and in-house quality control samples containing 60, 200, and 600 μg/L of CsA were prepared using blood samples from healthy donors.[20]

Sample Extraction

Before use, all glassware and plasticware were treated with AquaSil™ Siliconizing Fluid (Pierce, Rockford, IL). Saliva calibrators and patient samples were kept frozen at −20°C and thawed in a shaking water bath at 37°C. Patient or calibrator saliva samples (0.5 mL) were added to 1 mL of precipitant containing CsC in 1.5 mL polypropylene tubes. It is important that saliva be added to the precipitant because it was observed that recovery of CsA was greatly affected if the order of addition was reversed. After 5 minutes of standing, the samples were vortex mixed for 1 minute and centrifuged for 7 minutes at 15,000 *g*, and the supernatants were extracted using solid-phase extraction.

The C_{18} solid-phase extraction cartridges (200 mg, Waters, Milford, MA) were assembled on a manifold (Supelco Visiprep 24™ DL) (Bellefonte, PA). The cartridges were preconditioned with methanol and water (5 mL each). The supernatants were then added to the cartridges and left to drain under atmospheric pressure. Five minutes after all the supernatant had passed, the cartridges were washed sequentially with 5 mL deionized water, 2 mL of 50:50 vol/vol of methanol:water, and 2 mL heptane under atmospheric pressure. On passage of the heptane, the cartridges were subjected to vacuum at 20 psi for 15 minutes, followed by the elution of the analytes using 3 mL of 50:50 vol/vol solution of isopropanol:heptane. Five minutes later, any remaining eluting solution left in cartridges was extracted using vacuum for 1 minute and pooled with the main eluent. The eluents were then dried at 60°C in a centrifugal evaporator (Thermosavant, Holbrook, NY), reconstituted with 300 μL methanol, and vortex mixed for 5 minutes, and a 50-μL aliquot of this was injected into the HPLC column.

The blood assay was adapted from a method reported by Streit et al.[20] Whole blood calibrators, quality controls, or patient samples (0.1 mL) were vortex mixed for 30 seconds with 0.1 mL of a mixture of methanol and 0.3 mol/L $ZnSO_4$ (70:30 vol/vol) containing the internal standard CsC (250 μg/L) in 1.5-mL polypropylene tubes. The samples were centrifuged for 5 minutes at 15,000 *g*, and the supernatants were decanted. The samples were then recentrifuged for 1 minute at 15,000 *g*, and 150 μL was injected into the column.

HPLC Apparatus and Conditions

For saliva and blood assays, chromatography was performed on a Perkin Elmer series 200 liquid chromatography system consisting of an autosampler and 2 micropumps (Norwalk, CT). The analytic column consisted of an Aqua Perfect (150 × 3.0 mm; 5 μm) C_{18} reversed-phase column (MZ Analysentechnik, Germany) maintained at 65°C using a column heater (FC-Flatron, Milwaukee, WI). A precolumn filter (Supelco, 1/16-inch, Peek 2 μm Frit, Bellefonte, PA) was used throughout the analyses.

For the saliva assay, the mobile phase consisted of a 97:3 vol/vol mixture of methanol and 30 mmol/L ammonium ac-

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etate maintained at a flow rate of 0.5 mL/min. The run time was 5 minutes, and an equilibration step of 1.5 minutes was included. For the whole-blood assay, the column was washed for 1 minute at a flow rate of 0.6 mL/min with an 80:20 vol/vol mixture of methanol and 30 mmoL/L ammonium acetate solution, followed by a 4-minute elution step at 0.6 mL/min with a 97:3 vol/vol mixture of methanol:30 mmoL/L ammonium acetate. The mobile phase was then converted for 0.5 minutes to 80:20 vol/vol mixture of methanol:30 mmoL ammonium acetate in preparation for the next injection.

Mass Spectrometer Conditions

The API 2000 Triple Quadrupole Mass Spectrometer (LC-MS/MS system) (Sciex, Toronto, Canada) equipped with a turbo-ion spray source was used for all analyses. High-purity nitrogen gas obtained from a 240-L Liquid Nitrogen Dewar (Medford, MA) was used as nebulizer (Gas 1), auxiliary (Gas 2), and collision gases. For multiple reactant monitoring (MRM), the ammonium adducts $[M + NH_4]^+$ of CsA and CsC in the first quadrupole (Q_1) and their deaminated protonated adduct ions $[M + H]^+$ in the third quadrupole (Q_3) were selected. System control and data acquisition were performed using the Analyst Service Pack version 1.2 software.

Source parameters including curtain gas pressure, temperature, ion spray voltage, and gas 1 and 2 pressures were optimized by flow injection analysis. Compound parameters including declustering potential, focusing potential, entrance potential, collision cell entrance potential, collision energy, and collision cell exit potential were optimized by infusing a 1 mg/L solution of CsA and CsC in mobile phase at a flow rate of 0.01 mL/min into the mass spectrometer. The flow injection analysis yielded the optimized source parameters of curtain gas: 10 psi, ion spray voltage 4500 V, temperature 350°C, gas 1 20 psi, and gas 2 40 psi. Ramping in the MRM mode further improved the sensitivity for both CsA and CsC ion pairs. A low declustering potential (< 10 V) favored the entry of the ammonium fragment $[M + NH_4]^+$ for both CsA and CsC into the first quadrupole (Q_1). Accordingly, the ramped parameters in MRM mode included a declustering potential (DP) 6 V, focusing potential (FP) 392 V, entrance potential (EP) 10 V, collision energy 40 V, and a collision cell exit potential 30 V for CsA and CsC.

Method Validation

Assay validation for determination of CsA in saliva was performed according to the FDA guidelines for Bioanalytical Methods Validation for Human Studies.[19] The lower limit of quantification (LLOQ) was defined at a signal-to-noise ratio of 10:1 and limit of detection (LOD) at signal-to-noise ratio of 5:1. The calibration curve was constructed by injecting triplicates of the extracted saliva-based calibrators inclusive of the LLOQ and the quality controls on 6 different study days. The CsA concentrations on the calibration curve ranged from 1 to 300 μg/L, and the quality controls were 2, 75, and 200 μg/L. A $1/x^2$ weighted regression was used to fit the calibration data. The accuracy and interday coefficient of variation (%CV) of the method over the analytic range were determined from the back-calculated results obtained using the regression equation for each calibration curve on each of the 6 days.

The intraday %CV of the method was determined by measuring the CsA concentration in the quality control samples in 3 batches. This was repeated on 2 consecutive days. The interday %CV and inaccuracy were based on quality control concentration results obtained on at least 6 different days. The preset level of inaccuracy and imprecision of the assay method was set to be ≤ 15% for calibrators and QCs, except for the LLOQ, for which it could be ≤ 20%. The recovery of the analytes from saliva or blood matrix was determined by comparing the peak areas of the extracted samples, spiked with CsA and CsC against nonextracted CsA and CsC solutions.

Testing for freeze/thaw stability (3 cycles of freezing at −20°C and thawing at room temperature [25°C] were performed on 3 aliquots each of 3 quality control samples) and short-term room temperature stability (3 aliquots of each of the quality control samples maintained at room temperature for 4 hours before sample extraction) of CsA in saliva were conducted according to the FDA guidelines.[21]

RESULTS

The mass transitions selected in the MRM were *m/z* 1219.9 → *m/z* 1202.9 for CsA and *m/z* 1235.9 → *m/z* 1218.9 for CsC. The retention times for CsA and CsC were approximately 2.4 and 2.2 minutes, respectively. The specificity of the assay indicated no interfering peaks at the retention time of CsA in blank saliva (Fig. 1A) or blank saliva spiked with internal standard (Fig. 1B). The limit of detection was 0.6 μg/L, and the LLOQ was 1.0 μg/L with a signal-to-noise ratio of 10:1.

During the initial stage of method development, CsD was used as the internal standard for cyclosporine. It was observed that the samples of CsD were contaminated with CsA at a level of 0.2 μg/L (Fig. 2). The contamination was reported to Novartis, and subsequently CsC was used as the internal standard. No trace of CsA was found in CsC samples.

The calibration curve was linear over the working concentration range of 1–300 μg/L with correlation coefficient values ranging from 0.9732 to 0.9968. The mean extraction recovery over the concentration range 1–300 μg/L was approximately 84.7 ± 2.6% for CsA (Table 1) and approximately 93.7 ± 4.4% for CsC. The accuracy and intra- and interday coefficients of variation (%CVs) are expressed in Table 1. The accuracy for the low (2 μg/L), medium (75 μg/L), and high (200 μg/L) quality controls (QCs) ranged from 92.0% to 104.7%. The intra- and interday %CVs for the QCs ranged from 6.9% to 12.2% and 8.3% to 12.1%, respectively. Both intra- and interday %CVs were within 15%, the accept-

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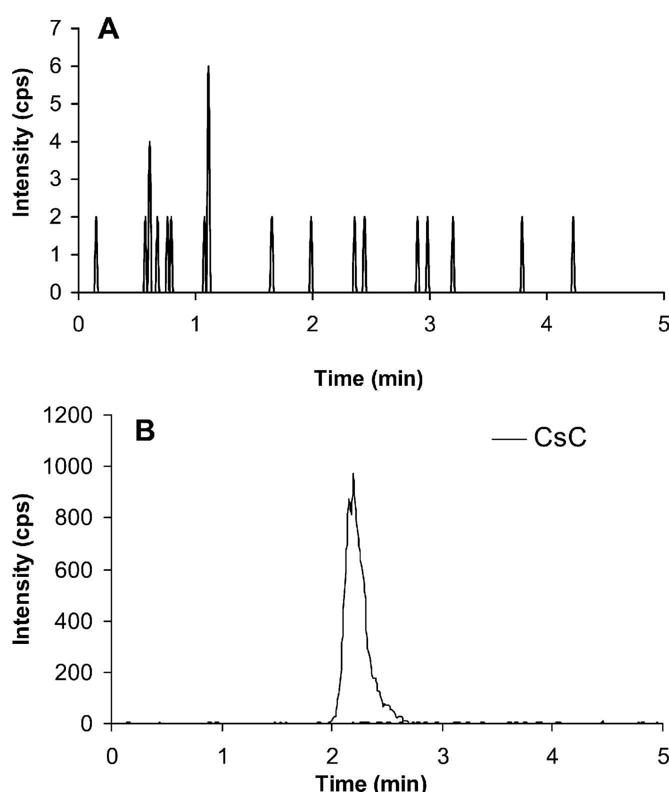

FIGURE 1. A, Blank extracted saliva shows no interference with cyclosporine (CsA) or cyclosporine C (CsC). B, Chromatogram of CsC (20 μg/L) extracted from saliva with no CsA added.

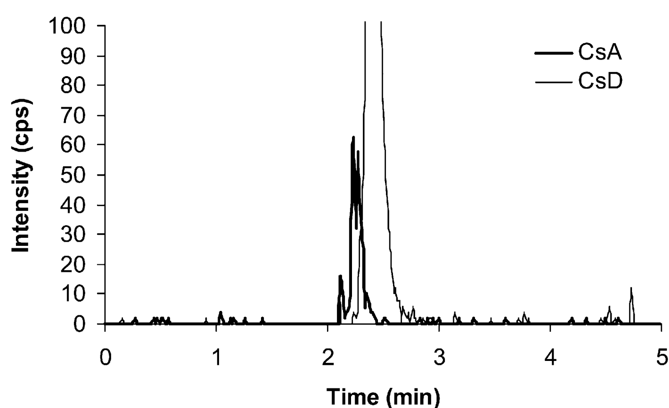

FIGURE 2. Chromatogram of cyclosporine D (0.05 μg/L) that shows contamination with cyclosporine A.

able limit according to FDA guidelines.[19] The accuracy and CVs of freeze/thaw and short-term stabilities are shown in Table 2. No systematic loss of sensitivity was observed in the absolute peak area measures as a result of sample storage.

Paired saliva and blood samples from 15 kidney transplant recipients were analyzed using the validated LC-MS/MS method. The results of measured saliva and blood concentrations are depicted in Figure 3. No CsA concentration was detectable in either saliva or blood samples of 1 of the 15 patients. A reasonable agreement (correlation coefficient = 0.695; P = 0.006) was observed between the saliva and blood concentrations in the remaining 14 patients.

DISCUSSION

We report for the first time the development and validation of a sensitive and specific LC-MS/MS method for determination of CsA in saliva. Therapeutic drug monitoring in saliva offers many advantages over a blood- or plasma-based method. No venipuncture is required as is the case with blood collection, and it can be performed, with minimal training, by the patient or a caregiver. The fingerprick method of blood collection seems to be a better alternative to venipuncture; however, it still remains invasive, and it also may be difficult to obtain sufficient quantity of blood from some patients.[22] Saliva monitoring requires small amounts of sample (less than 1 mL) and is ideal for drug monitoring in children and patients with difficult venous access. Drugs enter saliva predominately *via* passive diffusion, a process that is limited to lipophilic and unionized drugs. The diffusion of drugs between blood and saliva is also limited to the unbound fraction of the drug because the "protein-bound drug complex" is unable to pass

TABLE 1. Recovery, Accuracy, and Imprecision of the LC-MS/MS Assay

Quality Control Concentration (μg/L)	Extraction Recovery (%) (n = 6)	Accuracy (%) (n = 6)	CV% Intraday (n = 3)*	CV% Interday (n = 6)
2	86.7 ± 5.7	104.7 ± 8.2	12.2	8.3
75	85.7 ± 9.3	103.5 ± 7.0	11.3	8.5
200	81.7 ± 8.0	92.0 ± 11.1	6.9	12.1

CV, coefficient of variation.
*Intraday CV was calculated using 3 batches of quality control concentrations on 2 consecutive days.

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TABLE 2. Results of Stability Studies

Quality Control Concentration (μg/L)	Stability Criteria: Freeze/Thaw* CV%	Freeze/Thaw* Accuracy (%)	Short-Term Stability† CV%	Short-Term Stability† Accuracy (%)
2	7.9	96.2	2.6	92.2
75	10.7	101.2	8.1	98.5
200	7.6	104.7	2.2	99.8

CV, coefficient of variation.
*Three freeze/thaw cycles were performed.
†The duration of time before extraction was 4 hours.

through small channels in the capillaries of salivary glands.[16] It is therefore conceivable to believe that the salivary concentration will reflect the unbound and pharmacologically active species of a drug.

The unbound concentration of CsA is believed to be responsible for its immunosuppressive action.[9] However, it is practically impossible to measure the CsA unbound concentration in plasma or blood because of difficulties associated with the measurement of unbound concentration of such lipophilic molecules. It has been speculated that saliva monitoring is ideal for measuring neutral lipophilic compounds such as CsA.[23] The availability of a saliva-based method is therefore essential to measure the salivary concentration of CsA and to explore its correlation with the total or unbound concentration.

To date the only reported assay for measuring CsA in saliva was a polyclonal [^{125}I]cyclosporine radioimmunoassay (RIA) method modified from a Sandoz kit by Coates and colleagues.[24] This method was based on a kit that is no longer commercially available. The major disadvantage of this method was nonspecificity because of cross-reactivity with CsA metabolites and the requirement to produce [^{125}I]CsA to replace the original [^{3}H]CsA. Mass spectrometry (MS) coupled with HPLC is increasingly used for the measurement of CsA in whole blood. The LC-MS/MS methodology offers many advantages to an immunoassay based method including superior sensitivity, specificity and a rapid turnaround time. The LC-MS/MS technique was therefore used in the development of an assay for CsA measurement in saliva.

Saliva measurement of CsA presented a number of challenging issues that had to be overcome for successful method development. First, the concentration of CsA in saliva would be much lower than the CsA concentration in whole blood. In a study of 36 kidney transplant recipients, the mean ± SD of trough concentration of CsA in saliva was 8.3 ± 5.2 μg/L using the RIA method described earlier.[24] This concentration is much lower than the expected trough concentrations in whole blood. With use of the ammonium adducts for CsA and CsC, a LLOQ of 1 μg/L, which is more suitable for measurement of CsA in saliva, was obtained.

The next challenge was to develop an efficient method for extracting CsA from saliva. Because of the viscous and gluey nature of the saliva matrix, the recovery of CsA extraction was around 20% using liquid-liquid or solid-phase extraction methods previously reported for blood assays. Addition of the matrix to the protein precipitant solution significantly improved the recovery of CsA extraction. We have also observed that silanizing all glassware and plasticware increased the extraction efficiency.

Almost exclusively previous mass spectrometry methods for CsA used CsD as internal standard.[20,25,26] Keevil and colleagues[25] also used the commercially available molecule, ascomycin as the internal standard for CsA. Originally we had used CsD, during which we noticed that CsD was contaminated with CsA (Fig. 2). The CsA area under the peak accounted for almost 10% of the CsD area under the peak for 0.05 μg/L of CsD. This contamination has not been reported previously in the literature. Novartis Pharmaceuticals was informed about the contamination, and they kindly donated a sample of CsC that showed no contamination with CsA. Although this contamination may not be problematic for HPLC analysis, it can potentially generate erroneous measurements near the assay LLOQ for the LC-MS/MS. Our attempts to use ascomycin as the internal standard did not produce adequate recovery because of the differences between the molecular structures of ascomycin and CsA. In future we recommend that CsC be used instead of CsD to avoid introducing bias in calculation of unknown CsA, especially around the LLOQ concentrations.

In this study CsA concentrations in blood and saliva from 15 kidney transplant recipients were measured. The purpose of these measurements was to test the LC-MS/MS method and to obtain a crude approximation of the correlation between CsA concentrations in blood and saliva. Because the original intention was to have a wide range of concentration,

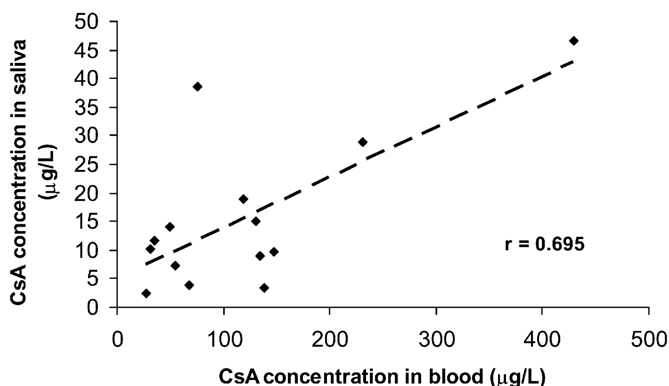

FIGURE 3. Plot of cyclosporine concentrations in whole blood versus saliva from 14 kidney transplant recipients.

no stipulation on time of sampling was implemented as long as the blood and saliva samples were obtained within a 10-minute time period. Although little can be concluded from such a small sample size, the saliva and blood concentrations broadly correlate with each other. Also, this study included no documentation on the existence of gingival hyperplasia in the patients. This condition, which is one of the most important side effects of CsA therapy, is associated with an increased plaque accumulation[27] and bleeding in mouth,[28] which may lead to erroneous measurement of CsA concentration in saliva. Because the existence of traces of whole blood in saliva can overestimate the salivary concentration of CsA, in any future study of CsA saliva determination it is imperative to document the traces of blood in saliva.

In this study we have collected nonstimulated saliva from kidney transplant recipients. The stimulation of salivary flow by placing citric acid on the tongue or chewing paraffin wax may generate more saliva in a shorter period of time.[18] However, the potential problems of changes in the salivary pH and adsorption to the paraffin wax must be evaluated for any specific drug before this technique can be employed in clinical studies. In addition, it is theoretically possible that plasma and saliva equilibration may not be achieved when a larger volume of saliva is produced in a short period of time; therefore, the saliva concentration may not represent the true saliva-to-plasma concentration ratio.[18] Currently, we are not familiar with the effects of stimulation on the salivary concentration of CsA; however, the CsA concentration in the stimulated saliva must be compared with the nonstimulated saliva in the context of a clinical study.

At the present time no therapeutic range is available for the CsA concentration in saliva. As a future direction for this study, we must first evaluate the degree of correlation between saliva with total and unbound concentrations of CsA in plasma. In addition, we have to obtain saliva samples in parallel to the blood and plasma samples in a conventional pharmacokinetic study and establish the concentration–time relationship for the saliva CsA concentration. Once these issues are resolved, a longitudinal clinical study must be performed to establish the correlation between salivary CsA concentration and organ rejections or CsA side effects.

CONCLUSION

In conclusion, a sensitive, specific, precise, and accurate method for the measurement of CsA in saliva is reported. The relevance of saliva concentration monitoring in CsA therapeutic drug monitoring requires further investigation, especially in pediatric patients, patients with difficult venous access, and patients suffering from gingival hyperplasia. Additionally, to establish the usefulness of the salivary concentration of CsA in predicting its unbound concentration, the saliva concentration must be measured in parallel with the unbound plasma concentration.

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