Simultaneous Quantification of Cyclosporin A and its Major Metabolites by Time-of-flight Secondary-ion Mass Spectrometry and Matrix-assisted Laser Desorption/Ionization Mass Spectrometry Utilizing Data Analysis Techniques: Comparison with High-performance Liquid Chromatography

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Simultaneous quantification of cyclosporin A (CsA) and its major metabolite (AM1) in blood has been achieved using time-of-flight secondary-ion mass spectrometry (TOF-SIMS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Previous investigations indicated that spectral interferences exist in the analysis of CsA blood samples by the above methods. In TOF-SIMS, interference is caused by overlap of the Ag-cationized internal standard, cyclosporin D (CsD), with the Ag-cationized metabolite, AM1. To resolve this interference and obtain quantitative information, cross-correlation analysis was applied to the TOF-SIMS data. Application of damped non-linear least squares curve-fitting was carried out to resolve an interference in the MALDI-TOF-MS data due to multiple cationization products (i.e. Na and K).

Measurement of standard samples indicates that the minimum accuracy (95% confidence level) of the TOF-SIMS method was better than 9% for CsA and 13% for AM1 using only one standard curve. Similarly, the minimum accuracy of the MALDI-TOF-MS method was determined to be 14% for CsA and better than 25% for AM1. Blood samples obtained from transplant patients receiving CsA were analyzed by polyclonal fluorescence polarization immunoassay, high-performance liquid chromatography (HPLC), and by both TOF-MS methods. Both TOF-MS results for CsA and mono-hydroxylated CsA are in good agreement with the HPLC results.

INTRODUCTION

Monitoring the blood or plasma concentrations of immunosuppressants remains an important part of the use of these agents following organ transplantation. The most common immunosuppressant in use today is cyclosporin A (CsA), with newer agents such as FK-506 which has recently been approved, cyclosporin G (CsG) and rapamycin undergoing clinical trials. Blood concentrations of CsA are often monitored to minimize toxicity, prevent graft rejection, and to monitor compliance with drug therapy. The presence of a large inter-and intra-individual variation in the pharmacokinetics of CsA, which is most pronounced in liver transplant recipients, necessitates individualization of the CsA dosing regimen.

Routine blood-level monitoring of immunosuppressants is accomplished by several different analytical methods such as polyclonal radioimmunoassay (PC-RIA), fluorescence polarization immunoassay (FPIA), and enzyme-linked immunosorbent assay (ELISA), which have been extensively evaluated for organ transplant patients; their use in comparison to high-performance liquid chromatography (HPLC) has been assessed. Both PC-RIA and FPIA methods overestimate CsA levels as the antibody used cross-reacts with the metabolites of CsA that are present in plasma or blood. One study showed that FPIA overestimated CsA concentrations by 51-132%, compared to HPLC. Later investigations demonstrated that whole blood samples from liver transplant patients contained amounts of AM1 and other CsA metabolites.
that are in excess of concentrations observed in other transplant populations.18 The nomenclature used throughout this paper is that proposed by Yatscoff et al.21 The mono-hydroxylated metabolite occurring at amino acid 1 was previously referred to as M-17; however, the new nomenclature for CsA metabolites provides information on the chemical structure, i.e. M-17 is now referred to as AM1.) The ratio of PC-RIA/HPLC and FPIA/HPLC changes over a period of time and is a function of the time of blood sampling in relationship to drug administration, the absolute concentration of the drug, and interactions of other drugs with CsA. The role of CsA metabolites in overall immunosuppression and toxicity is still a controversial issue.19 Newer immunosuppressants, such as FK-506, have exhibited activity and/or toxicity but remain largely uninvestigated.18-20 At least one presumed FK-506 metabolite exhibits significant immunosuppressive activity which would necessitate routine monitoring of the parent drug and this metabolite.21

The use of mass spectrometry (MS) to characterize immunosuppressants and their metabolites has been accomplished using fast atom bombardment (FAB/MS),22-26 liquid chromatography mass spectrometry (LC/MS),29-32 tandem mass spectrometry (MS/MS),33 time-of-flight secondary-ion mass spectrometry (TOF-SIMS)34-36 and matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI/TOF-MS).37 A comparison between FAB/MS and TOF-SIMS for the analysis of biomolecules concluded that the latter had detection limits an order of magnitude better.37 The use of MS/MS utilizing an electrospray ion source to elucidate the structure of CsA metabolites has been demonstrated.38

Fundamental aspects concerning the quantification of biomolecules by TOF-SIMS38 (i.e. surface coverage, Poisson corrections, effect of sample preconcentration) and MALDI19 have been reported elsewhere. This paper deals with further quantitative investigations using both TOF-MS methods applied to a real biological system with validation by an external established technique (HPLC).

TOF-SIMS and MALDI/TOF-MS have recently been demonstrated for the quantification of CsA blood concentrations in transplant patients.33 Use of the TOF-SIMS method for determination of parent CsA presented no problem; however, since CsA is preferentially cationized with Ag, an interference of the isotopic patterns of the internal standard, cyclosporin D (CsD) and AM1 was observed. This limited the usefulness of the TOF-SIMS method because it could not simultaneously quantify AM1 without prior fractionation by HPLC. This interference could be overcome by: (1) using isotopically pure silver, but the cost is prohibitive; or (2) changing the internal standard (many cyclosporin analogues are available). The latter was not done because this investigation represents a model system and is explained in more detail, vide infra.

The MALDI/TOF-MS analytical method developed for CsA quantification was of limited applicability to samples from transplant patients due to overlapping Na and K adducts of each component (parent drug and metabolite) which did not allow adequate separation of CsD and AM1 at low levels. The application of damped non-linear least squares curve-fitting (NLLSCF) was carried out to resolve mathematically this interference and subsequently allowed the quantification of CsA; however, the quantification of AM1 using NLLSCF was not thoroughly investigated in a systematic study.

This paper presents the use of cross-correlation analysis (CCA) for TOF-SIMS data and NLLSCF for MALDI/TOF-MS data to enable the techniques to simultaneously quantify CsA and AM1 in synthetic samples, and CsA and all mono-hydroxylated metabolites in allograft recipient samples. The results obtained from transplant samples are compared with gradient HPLC results which can measure CsA and some metabolites simultaneously. While it is realized that quantification of CsA metabolites may be unnecessary, this investigation serves as a model system to illustrate the usefulness of simple data analysis techniques for TOF-MS data to obtain quantitative information without prior fractionation by HPLC. CsA was chosen as a model system for two reasons. First, because the mass separation between CsD and AM1 is 2 Da, it is often the case that demethylated [M - 14 Da] and dehydroxylated [M - 16 Da] and/or hydroxylated [M + 16 Da] and methylated [M + 14] metabolites are present together. Second, the CsA system is the most extensively characterized to date, with its metabolic pathways being well characterized; thus, standards of suitable purity are available.

**EXPERIMENTAL**

Reagents and materials

Cyclosporin A and D were obtained from Sandoz Corporation (Basel, Switzerland). Tracemetal grade nitric acid was obtained from Fisher Scientific. Preparation and purification of AM1 has been described elsewhere.40 The high purity (99.9985%) silver foil used in this study was obtained from Alfa. All solvents used in HPLC analysis (water, acetonitrile, isopropyl alcohol and methanol) were HPLC grade and were purchased from Fisher Scientific.

**Instrumentation**

FPIA. This assay was performed according to the procedure described in the text supplied by Abbott Laboratories.41

HPLC. The chromatograph used in this work was a Hewlett Packard HP1050 gradient HPLC equipped with a column oven and a Hewlett Packard variable wavelength detector. Detection was carried out at 214 nm. Samples were reconstituted in MeOH prior to injection onto a 4.6 x 150 mm, 5 µm, C4, column purchased from Supleco. The column temperature was held constant at 70°C with a flow rate of 1.0 ml min⁻¹. The gradient profile started with 49% ACN (Solvent A) and 51% H2O (Solvent B) and was ramped linearly to 59/41 (A/B) at 33 min. At 37 min the solvent composition was...
The relative sensitivity factors (k) for AM1, AM1c and AM9 (relative to CsA) are 0.721, 0.615 and 0.566, respectively.

A pulsed Ar⁺ ion beam was used with a pulse length less than 1 ns, a variable spot diameter between 5 and 50 μm and a pulsed current of 0.5–1.0 pA. Secondary ions generated by the primary ion pulse were accelerated by a 3 keV extraction lens into the 1.87 m flight tube operated in the reflecting mode. The ions were post-accelerated to 10 keV prior to detection by a channelplate-scintillator–photomultiplier combination detector. The spectra were accumulated in the single-ion counting mode with a maximum of 256 stops/pulse and a time resolution of 472 ps/channel. The samples were mounted on a holder (maximum capacity of 24) that can be accessed using a high-precision XYZ manipulator.

Processing of data was done using in-house generated software and included mass calibration, measuring the (M + 107Ag)⁺ peaks of CsA and CsD, and application of cross-correlation analysis to determine the AM1/CsD ratio. The peak areas were measured with the integration limits determined by full width at half maximum (FWHM) using a linear background. Typical resolution (M/ΔM) at m/z 1000 is 8000 (FWHM).

MALDI/TOF-MS. The instrument employed in these investigations was a modified LAMMA 1000 (Leybold-Heraeus GmbH) mass spectrometer. The modification involved replacement of the laser, from an Nd-YAG to a Nd laser (VSL 337ND; Laser Science Inc., Boston, MA) emitting at 337 nm. Laser irradiance was approximately 5.0 × 10⁷ W cm⁻², post-acceleration 15 kV, with an XYZ manipulator and microscope to control the interaction of the laser with the sample. The instrument has been described elsewhere in more detail.

The multicomponent matrix employed in this study (2,5-dihydroxybenzoic acid (DHB) (20 mg ml⁻¹): 5-methoxy salicylic acid (MSA) (0.5 mg ml⁻¹):fucose (2 mg ml⁻¹)) was mixed with each sample in a volume ratio of 1:1 using a vortex-mixer. A 1 μl aliquot of this mixture was deposited on polished stainless steel substrates and dried in high purity N₂. Typically, analyte:matrix molar ratios of 1:10³ to 1:10⁵ were used. Several spectra (100–200) were added together to improve the S/N ratio. The analytical protocol for MALDI quantitative analysis has been described elsewhere.

Processing of data included spectral smoothing and mass calibration, followed by application of a curve-fitting routine to determine the (CsA + Na)⁺/(CsD + Na)⁺ and (AM1 + Na)⁺/(CsD + Na)⁺ ratios which is described, *vide infra*. Typical resolution (M/ΔM) at m/z 1200 is 800 (FWHM).

Sample preparation

CsA standard curve. The CsA standard curve was prepared using methanol solutions of CsA and CsD; ten samples with different CsA concentrations (0, 25, 50, 100, 200, 250, 500, 1000, 1375 and 2000 ng ml⁻¹) were used. CsD was added to all samples at 625 ng ml⁻¹. To enable use of the CsA standard curve to quantify AM1, the relative ionization efficiencies of CsA and AM1 had to be determined. To accomplish this, three samples were prepared in MeOH, each containing 1 μg ml⁻¹ CsA and 1 μg ml⁻¹ AM1.

To ensure that the presence of AM1 did not compromise the measurement of CsA, eight samples were prepared with CsA, CsD and AM1 present. CsD concentration was held constant at 625 ng ml⁻¹. Four samples contained 300 ng ml⁻¹ CsA and four samples contained 1 μg ml⁻¹ CsA, both with varying amounts of AM1 (0–1 μg ml⁻¹). The accuracy and precision of both TOF-MS methods was determined from the four samples for each concentration. The accuracy is reported as the relative error (RE). RE is the absolute error expressed relative to the size of the measured quantity. The precision is expressed as the coefficient of variation (CV). CV is the standard deviation expressed as the percentage of the mean.

AM1 standard sample. To determine the accuracy and precision of the TOF-MS methods to quantify AM1 using data analysis techniques, ten samples were prepared in MeOH, containing varying amounts of CsA (200 ng ml⁻¹–1 μg ml⁻¹), all containing 625 ng ml⁻¹ CsD. AM1 was added at concentrations of 0, 150, 300, 600 and 1000 ng ml⁻¹; each concentration was prepared in duplicate.

Transplant sample. Ten blood samples from transplant patients on CsA were obtained from the University of Pittsburgh Medical Center. Either extraction of the blood samples was carried out, using a method previously described in detail, however, two modifications were made. First, 2 ml of blood was extracted (1 ml was used previously). Second, no pH adjustment was carried out prior to defatting the extract. The modifications to the extraction method increase the recovery of AM1. Samples were reconstituted in 150 μl of mobile phase prior to analysis. Measurement of each of these samples was then carried out using both the HPLC and TOF-MS methods.

Data analysis techniques

Cross-correlation analysis (CCA). CCA was carried out using normalized composite theoretical isotopic distributions (TIDs) to represent the CsD and AM1...
signals. This acts as a fingerprint, not only of the species present (qualitative), but also their relative amounts (quantitative). Cross-correlation compares the normalized experimental spectrum with each of a series of composite TIDs, which consists of 101 spectra representing CsD (0-100%) with AM1 (100%-CsD%). CCA determines the correlation coefficient, \( r_0 \), which was normalized between -1.0 and +1.0. The highest \( r_0 \) indicates the best agreement between the experimental and theoretical data. The most important step in determining precise results using CCA is proper calibration of the experimental spectrum. After a system is defined and tested with several samples, CCA analysis can be carried out automatically in a few minutes. Essentially identical results can be obtained with principal component regression (PCR), where the composite series represents a calibration set.\(^{47}\)

**Non-linear least squares curve-fitting (NLLSCF).** Each MALDI/TOF-MS spectrum was fitted using six different spectral groups, each group representing a different molecular species. A given group represents the TID of that particular species which provides well established theoretical knowledge about the position (mass) and the intensity ratios. The six groups used in the curve-fitting procedure are listed in Table 1. Use of six groups was necessary because cationization of each species with Na and K is hard to control.

The mass of each group was set at the appropriate value listed in Table 1 and the FWHM were set at the experimentally determined value of ~0.8 Da. The peak position, FWHM, and area ratios were then allowed to float (with defined limits) during calculation. The results of the optimized curve-fit were compared with the theoretical values. Accurate peak separation, rather than exact mass prediction, was of importance in the NLLSCF. Thus, unlike CCA, poor calibration does not have any influence on the NLLSCF results, particularly with the MALDI/TOF-MS spectra where mass calibration is problematic. The peaks were fit using a Voigt function with 99% Lorentzian character. This function correlated well with the experimental peak shapes and also somewhat compensated for the background.

NLLSCF also generates residual spectra, which are the difference between the experimental data and the theoretical prediction of the data. A relative measure of residual intensity is given by the percentage residual excursion, \( REX\% = 100 (R_{\text{max}} - R_{\text{min}}) / (Y_{\text{max}} - Y_{\text{min}}) \), where \( R_{\text{max}} \) and \( R_{\text{min}} \) are the maximum and minimum values of the residuals, and \( Y_{\text{max}} \) and \( Y_{\text{min}} \) are the maximum and minimum of the experimental data. Lower REX\% values indicate better agreement between the experimental and the theoretical spectra. In addition, the residuals offer qualitative information, for example, a positive low frequency residual may indicate the presence of another component.

## RESULTS AND DISCUSSION

### CsA standard curve results

The plot of the ratio of the peak integral values \((\text{CsA} + ^{107}\text{Ag})^+ / (\text{CsD} + ^{107}\text{Ag})^+\) vs. CsA concentration obtained from TOF-SIMS is shown in Fig. 1. The data were fit using linear least squares regression analysis (LLSRA); a linear response was obtained over the concentration region investigated. The scatter limits of the data, also shown in Fig. 1, allow one to detect possible outliers. At a confidence limit of 95% with \( N = 60 \), no more than three points should lie outside this range. As seen in Fig. 1, no points were determined to be outliers. The equation of the line shown in Fig. 1 is

\[
y = 1.93E - 03x + 0.092, \quad \text{where } y \text{ is the intensity ratio and } x \text{ is the concentration of CsA in ng ml}^{-1}.\]

The relative standard deviation (%RSD) of the slope was 0.54% with a correlation coefficient \( r_0 \) of 0.999. This curve was used to measure CsA in all standard samples and in samples obtained from transplant patients.

The plot of the ratio of the peak integral values \((\text{CsA} + \text{Na})^+ / (\text{CsD} + \text{Na})^+\) vs. CsA concentration obtained from the MALDI/TOF-MS method was also fit using LLSRA. The resultant equation of the fitted line was 

\[
y = 2.35E - 03x + 0.0658, \quad \text{where } y \text{ is the intensity ratio and } x \text{ is the concentration of CsA in ng ml}^{-1}.\]

The %RSD of the slope was found to be 1.36% and \( r_0 \) = 0.997. A similar MALDI/TOF-MS standard curve for CsA has been reported elsewhere.\(^{35}\)

### Table 1: Species used to model MALDI/TOF-MS data using non-linear least squares curve-fitting

| Group no. | Assignment | Nominal mass (Da) |
|-----------|------------|------------------|
| 1         | CsA + Na   | 1225             |
| 2         | Impurity*  | -                |
| 3         | CsD + Na   | 1239             |
| 4         | CsA + K    | 1241             |
| 5         | AM1 + Na   | 1241             |
| 6         | CsD + K    | 1256             |
| 7         | AM1 + K    | 1257             |

* Modeled as a group 1 species + 2H.

### Figure 1. TOF-SIMS standard curve for cyclosporin A (25-2000 ng ml\(^{-1}\)) using cyclosporin D as the internal reference material (625 ng ml\(^{-1}\)). The result of applying LLSRA to the experimental data (■) is shown by the solid line, with the scatter limits shown by the dashed line.
Relative ionization efficiencies of CsA and AM1

TOF-SIMS. TOF-SIMS spectra of CsA and AM1, both at 1 µg ml⁻¹, are shown in Fig. 2. The spectrum shown in Fig. 2A was obtained from an etched silver substrate with no NaCl added. The spectrum exhibits weak Na adducts of each component, with dominant peaks being the Ag-cationized species. (The nomenclature used in this paper for the relative ionization efficiencies of CsA and AM1 (CsA/AM1) is the type of ion formed (i.e. H, Na, K or Ag) followed by RIE, for example, the relative ionization efficiency (RIE) of the Ag adduct of CsA to AM1 is referred to as Ag-RIE.) The Ag-RIE was obtained on three different samples and five spectra were collected for each sample, resulting in 15 spectra being used to determine the average Ag-RIE; Ag-RIE = 4.45 ± 0.578, reported as the mean the confidence interval of the mean at the 95% confidence level (C1M5%). The RIE of CsA and AM1 with Ag (Ag-RIE) in Figure 2A was determined to be 4.72. Figure 2B illustrates the effect of NaCl addition to the silver substrate on the TOF-SIMS spectrum of the same sample. Note that the Na-cationized peaks are less intense than the Ag adducts. The RIE for the Ag adducts did not change statistically in this spectrum and was found to be 4.88. The Na-RIE was obtained from 15 spectra and the average value was determined to be 1.47 ± 0.193, a distinct difference. The RIE for the Na-cationized species (Na-RIE) in Fig. 2B was determined to be 1.63.

MALDI/TOF-MS. To determine an accurate value of the Na-RIE from MALDI/TOF-MS spectra, one has to account for the ([CsA + K])⁺ contribution to the peak centered at 1241 Da. The extent to which cationization occurs with K is hard to control owing to different levels of contamination. This is the reason for including the ([CsD + K])⁺ peak in the analysis of the MALDI/TOF-MS data. As shown in Table 1, the area of group 4 is due to two components, (AM1 + Na)⁺ and (CsA + K)⁺. An empirical relationship allowed more accurate determination of the (AM1 + Na) peak area and is shown in Eqn (1).

\[
I(AM1 + Na)^+ \approx I(Mass 1241 Da)^+ \frac{[CsA]}{[CsD]} \times A(CsD + K)^+ \tag{1}
\]

where \(I(AM1 + Na)^+ = \) area of Na-cationized AM1; Mass 1241 Da = area of 1241 Da species from curve-fitting results; \([CsA] = \) concentration of CsA; \([CsD] = \) concentration of CsD; \(A(CsD + K)^+ = \) area determined from curve-fitting results.

Figure 3A shows a MALDI mass spectrum of a sample containing CsA and AM1, each at a concentration of 1 µg ml⁻¹. The dominant peaks in the spectrum are due to Na adducts of each component, with K adducts also present. The resolution of the spectrum is sufficient to allow peak positions to be defined unambiguously, which permits curve-fitting to be carried out more precisely. Figure 3B shows the curve-fitted mass spectrum of the sample shown in Fig. 3A. The dots are the experimental points and the line is the overall fit. The areas of groups 1 and 4 (Table 1) which are (CsA + Na)⁺ and (AM1 + Na)⁺, respectively, are determined from the NLLSCF results. The value of Na-RIE was determined from several samples and was found to be 1.45 ± 0.26. This is the same as the TOF-SIMS Na-RIE within experimental error. Without accounting for the (CsA + K)⁺ area using Eqn (1), the calculated...
value falls within the CIM \textsubscript{95\%} stated above (i.e. the [K\textsuperscript{+}] was minimal in these samples).

Additional uncertainties are present in the measurement of the (AM1/CsD) ratio (i.e. quantification of AM1) which are not introduced by curve-fitting of the mass spectra. These errors include: (1) error of CsA concentration determinations in unknown samples; (2) (CsD + K\textsuperscript{+}) peak area determinations; and (3) the use of an inaccurate value for Na-RIE (shown above). Thus, it is expected that the quantification of AM1 will be both less accurate and less precise than quantifying CsA.

**Quantification of CsA in standard MeOH samples**

To ensure that the accuracy and precision of quantifying CsA by TOF-SIMS was not compromised by addition of AM1, eight samples were analyzed containing mixtures of the two (four 300 ng ml\textsuperscript{-1} and four 1 \mu g ml\textsuperscript{-1}). The results are listed in Table 2. The accuracy (RE) was found to be 0.20\% for the 1 \mu g ml\textsuperscript{-1} standard and 3.7\% for the 300 ng ml\textsuperscript{-1} sample. The accuracy is expected to increase as the concentration increases. The minimum accuracy was calculated using the CIM \textsubscript{95\%} and was found to be better than 9\% in the same manner as reported previously.\textsuperscript{3,5} The CVs ranged from 2.4 to 4.5\%. The overall precision and accuracy achieved for these samples were similar to the results previously reported for blood extracts, where we achieved an accuracy of better than 6.0\% and a precision ranging from 3.5 to 5.0\%.\textsuperscript{3,5} Thus, the addition of AM1 did not compromise the CsA assay.

Similarly, the same standard samples measured by MALDI/TOF-MS resulted in an accuracy of 8.7\% for the 300 ng ml\textsuperscript{-1} sample and 1.8\% for the 1 \mu g ml\textsuperscript{-1} sample with CVs ranging from 4.1 to 9.3\%. The minimum accuracy calculated at the 95\% confidence level was 14\%. These results are summarized in Table 2. The accuracy and precision of these standard samples also agree with previously reported results;\textsuperscript{3,5} the presence of AM1 did not compromise the MALDI/TOF-MS method.

**Quantification of AM1 in standard MeOH samples**

The structures of CsA, CsD and some of the CsA metabolites have been reported elsewhere.\textsuperscript{1,6} The metabolites that are of relevance in this investigation, because of their similar molecular weights, are shown below with their exact masses: \textsuperscript{1,6}

| Metabolite | Nomenclature | Exact Mass |
|------------|--------------|------------|
| AM1        |              | 1217.84    |
| AM1c       |              | 1217.84    |
| AM9        |              | 1217.84    |
| AM4N69     |              | 1219.82    |

As stated, we cannot differentiate the metabolites by either TOF-MS technique. This investigation will quantify AM1 in blood samples obtained from transplant patients by HPLC and all mono-hydroxylated metabolites by both TOF-MS methods. However, AM1 is the major metabolite of CsA in blood and the concentrations of AM1 exceed those of AM1c and AM9 fivefold, so that TOF-MS results should be comparable to those from HPLC.\textsuperscript{1,6}

**TOF-SIMS.** A portion of the data matrix used in the cross-correlation analysis (CCA) along with two experimental spectra are shown in Fig. 4. Spectra 1–15 are the composite theoretical isotopic distributions (TIDs) for varying amounts of CsD and AM1, as listed. Spectrum A is for a standard sample with only CsA and CsD present and spectrum B is for a standard sample with 625 ng ml\textsuperscript{-1} CsD and 1 \mu g ml\textsuperscript{-1} AM1 present. Spectrum A showed the highest correlation coefficient (r\textsubscript{p}) with theoretical spectrum 1, which indicates that the only component present is CsD. In fact, for analysis of 30 standard samples containing only CsA and CsD, the experimental data always had the highest correlation with spectrum 1.
Spectrum B showed the best correlation with spectrum 9 which is a composite of 70% CsD and 30% AM1. The ratio of (AM1/CsD) is then calculated to be 0.4286. In order to convert this ratio into a concentration using the CsA standard curve, the following equation is used:

\[
\frac{[\text{AM1 + Ag}]}{[\text{CsD + Ag}]} = \frac{\%\text{AM1}}{\%\text{CsD}} \times \text{Ag-RIE}
\]

where \([\text{AM1 + Ag}]/[\text{CsD + Ag}] = \) corrected area ratio. All other variables were previously defined. Thus, the concentration of the AM1 standard samples can be determined using the CsA standard curve by multiplying 0.4286 (30/70) by 4.45 (Ag-RIE), which is 1.907. Using this intensity ratio (1.907) the concentration of the 1 pg ml\(^{-1}\) AM1 standard was determined to be 939 ng ml\(^{-1}\) using the CsA standard curve. Table 3 summarizes the CCA results of the five standard samples containing varying concentrations of AM1. The accuracy in determining AM1 concentrations using CCA ranged from 0 to 6.0% (RE), with CVs ranging from 0 to 10%; these are also listed in Table 3. The accuracy of this analytical method is slightly reduced when using CCA to quantify AM1, compared to the direct quantification of CsA. This may be attributed to: (1) an inaccurate value of Ag-RIE; and (2) the calculation of the ratio (AM1/CsD) using CCA. However, the accuracy and precision are still acceptable for clinical use.

MALDI/TOF-MS. Figure 5A shows a MALDI mass spectrum of a MeOH solution containing 300 ng ml\(^{-1}\) CsA, 625 ng ml\(^{-1}\) CsD and 600 ng ml\(^{-1}\) AM1. The experimental data were interpolated to decrease the number of points to 200. The number of points is reduced to allow the curve-fitting routine to be carried out in a timely manner. The interpolated experimental data and curve-fitted spectrum are shown in Fig. 5B. This spectrum displays all six groups (Table 1) with reasonable intensity; the first peak in each group is identified with an arrow. The results of this curve fit are summarized in Table 4. The mass separation \((\Delta m_n)\) is defined as \((\text{mass group } 2-6 - \text{mass group 1})\). The error in the mass separation is highest for group 2 (5.16%). Also note that the FWHM is smaller than for the rest of the groups of known origin, indicating that the impurity is not being correctly modeled as \((\text{CsA + 2H + Na})^+\). Attempts to identify the impurity have been unsuccessful; however, the impurity is only detected in the AM1 stock solution. The error in separation of the other groups is less than 0.5%, which is attributed to the smoothing of the data and decreasing the number of points.

Owing to the chemical similarity of the species investigated and the small mass range (50 Da) in which these

Table 3. Results of quantifying AM1 in standard samples containing CsA (200 ng ml\(^{-1}\)-1 µg ml\(^{-1}\)) and CsD (625 mg ml\(^{-1}\))

| Standard sample conc. (ng ml\(^{-1}\)) | Mean conc. (ng ml\(^{-1}\)) | CV (%) | Mean conc. (ng ml\(^{-1}\)) | CV (%) |
|--------------------------------------|-----------------------------|--------|-----------------------------|--------|
| TOF-SIMS                             | MALDI/TOF-MS                |        |                             |        |
| 0                                    | 0 (0)                       | 19     | 48.6 (—)                    |        |
| 150                                  | 145 (10.3)                  | 143    | 18 (4.7)                    |        |
| 300                                  | 312 (5.4)                   | 323    | 10 (7.7)                    |        |
| 600                                  | 564 (6.0)                   | 664    | 8.5 (9.0)                   |        |
| 1000                                 | 970 (3.5)                   | 1019   | 6.7 (1.9)                   |        |

Table 4. MALDI mass spectral results derived from NLLSCF of mass spectrum shown in Fig. 5(B)

| Group no. | Assignment                          | Peak position (Da) | Experimental \(\Delta m_n\) (Da) | Theoretical \(\Delta m_n\) (Da) | \% Error \((\Delta m_n - \Delta m) / \Delta m\) | FWHM (Da) |
|-----------|-------------------------------------|-------------------|--------------------------------|--------------------------------|----------------------------------|-----------|
| 1         | (CsA + Na) *                         | 1225.23           | —                               | —                              | —                                | 0.85      |
| 2         | (?) *                               | 1227.35           | 2.12                            | 0.016                          | 5.16                             | 0.77      |
| 3         | (CsD + Na) *                         | 1239.23           | 14.00                           | 14.016                         | 0.11                             | 0.85      |
| 4         | (CsA + K) *                          | 1241.26           | 16.03                           | 15.974                         | 0.35                             | 0.84      |
| and (AM1 + Na) *                    |                          |                   |                                |                                 | 15.995                          | 0.22      |
| 5         | (CsD + K) *                          | 1255.27           | 30.04                           | 29.990                         | 0.17                             | 0.85      |
| 6         | (AM1 + K) *                          | 1257.23           | 32.00                           | 31.968                         | 0.10                             | 0.94      |
components reside, it is not expected that the FWHM should increase, which is the case. The FWHM remains constant (±0.01 Da). As mentioned, mass calibration is not important because the peak positions are allowed to float (± 1 Da). However, it is essential that separation between the groups makes spectroscopic sense.

The results of quantifying AM1 in standard samples by MALDI are also shown in Table 3. The concentration is calculated using Eqn (1) to determine the 

$$\frac{[\text{AM1} + \text{Na}]}{[\text{CsD} + \text{Na}]} = \frac{I(\text{AM1} + \text{Na})}{I(\text{CsD} + \text{Na})} \times \text{Na-RIE}_{\text{CsA/AM1}}$$

where $I[M]^+$ = intensity of each species; $[\text{AM1} + \text{Na}]_i/[\text{CsD} + \text{Na}]_i$ = corrected area ratio; Na-RIE_{CsA/AM1} = relative ionization efficiency. This allows use of the CsA standard curve.

Note in Table 3 that the precision of the AM1 measurement increases dramatically with increasing concentration. This indicates that Eqn (1) is not accurately removing the (CsA + K)$^+$ contribution from group 4. Several approaches were tried to account for this interference, the one presented (Eqn (1)) being the most successful. This has limited the accuracy with which AM1 can be quantified but it should be stressed that this is not a result of applying the curve-fitting routine. The minimum accuracy with which AM1 can be quantified by the MALDI/TOF-MS method in concert with NLLSCF was determined to be better than 25% (95% confidence level).

Analysis of allograft recipient samples

Ten transplant samples were analyzed by fluorescence polarization immunoassay (FPIA), HPLC and by both TOF-MS methods. Figure 6A shows a TOF-SIMS spectrum of a sample obtained from a transplant patient (no. 1) indicating a low CsA concentration and metabolite levels below detectable limits. The HPLC data also indicated small concentrations (<50 ng ml⁻¹) of CsA metabolites in this sample, which is confirmed by the low CsA FPIA/HPLC concentration ratio of 1.3 (a ratio of 1.0 indicates no metabolites are present). Figure 7A shows a curve-fitted MALDI/TOF-MS mass spectrum of sample no. 1; this spectrum also indicates a low CsA level in the patient sample, with few metabolic products present. Refer to Table 1 for peak assignments. Thus, both TOF-MS methods are capable of detecting CsA and its metabolites.

Figure 6B shows a TOF-SIMS spectrum of sample no. 2. Note that several Ag-cationized species are present with reasonable intensity compared to sample no. 1 (Fig. 6A). These Ag-cationized species are attributed to CsA metabolites and are listed in Table 5 with their molecular masses and peak assignments. The HPLC data for sample no. 2 indicated extensive metabolism of CsA, which is also consistent with the high CsA FPIA/HPLC concentration ratio (3.2). Inspection of the MALDI/TOF-MS spectrum of sample no. 2 in Fig. 7B clearly indicates the presence of several CsA metabolites. Note that several metabolites have exactly the same mass; thus, the TOF-MS methods cannot distinguish between them. CCA could be applied to the TOF-SIMS mass spectral region where peaks from metabolites 6 and 7 (Table 5) occur, allowing elucidation of their individual concentrations.
The concentrations of CsA in the ten blood samples have been determined using FPIA, HPLC and both TOF-MS methods, and the results are summarized in Table 6. Note that the FPIA results are higher than those from HPLC and both TOF-MS methods as a result of the immunoassay technique quantifying CsA and cross-reactive metabolites (see above). The CsA concentration by HPLC for blood sample no. 6 is higher than the FPIA and both TOF-MS results, indicating possible interferences in the HPLC assay. Sample no. 10 did not produce a good quality chromatogram and, owing to limited sample, this measurement could not be repeated. A correlation plot of each TOF-MS method vs. HPLC of the eight non-suspect CsA determinations is shown in Fig. 8 with the linear least-squares regression analysis (LLSRA) shown for each data set. The correlation coefficient \( r_0 \) was determined to be 0.988, with the %RSD of the slope found to be 2.698% for the TOF-SIMS method with \( r_0 = 0.986 \) and \%RSD,\_slope = 2.888% for the MALDI/TOF-MS method. A correlation plot of TOF-SIMS vs. MALDI/TOF-MS resulted in \( r_0 = 0.973 \).

Application of NLLSCF and CCA to the TOF-MS data was carried out and allowed quantification of total mono-hydroxylated metabolites for each sample. HPLC data provide information regarding each mono-hydroxylated metabolite. Figure 9 shows the HPLC and TOF-MS data for transplant samples, indicating substantial metabolism of CsA. The CsA mono-hydroxylated metabolites detected by HPLC are separated by species in Fig. 9 and since neither TOF-MS method can mass resolve each of the hydroxylated metabolites, the total mono-hydroxylated metabolite concentration by TOF-MS is reported. The HPLC data were corrected for the relative absorbance factors (see Experimental section); however, owing to the lack of available purified metabolites, the assumption was made that all mono-hydroxylated metabolites have the same relative ionization efficiencies as AM1 in

**Table 5. Peak assignments of CsA metabolites detected by TOF-SIMS**

| Peak assignment no. | Molecular mass (Da) (Ag-cationized) | Species |
|--------------------|-------------------------------------|---------|
| 1                  | 1281                                | Doubly demethylated CsA + Ag |
| 2                  | 1295                                | Demethylated CsA + Ag         |
| 3                  | 1309                                | CsA + Ag                       |
| 4                  | 1323                                | CsD + Ag                       |
| 5                  | 1325                                | Hydroxylated CsA + Ag          |
| 6                  | 1339                                | Hydroxylated-methylated CsA + Ag |
| 7                  | 1341                                | Doubly hydroxylated CsA + Ag   |

**Table 6. Quantification of CsA in allograft recipient blood samples by HPLC, TOF-SIMS and MALDI/TOF-MS**

| Transplant sample no. | FPIA (ng mL\(^{-1}\)) | HPLC (ng mL\(^{-1}\)) | TOF-SIMS (ng mL\(^{-1}\)) | MALDI/TOF-MS (ng mL\(^{-1}\)) |
|-----------------------|------------------------|------------------------|---------------------------|-------------------------------|
| 1                     | 88                     | 67                     | 94                        | 60                            |
| 2                     | 948                    | 293                    | 264                       | 260                           |
| 3                     | 664                    | 320                    | 276                       | 284                           |
| 4                     | 840                    | 392                    | 363                       | 338                           |
| 5                     | 300                    | 172                    | 166                       | 150                           |
| 6                     | 147                    | 226                    | 99                        | 74                            |
| 7                     | 439                    | 204                    | 186                       | 197                           |
| 8                     | 278                    | 156                    | 129                       | 120                           |
| 9                     | 1109                   | 407                    | 336                       | 414                           |
| 10                    | <50                    | —                      | 58                        | 61                            |

**Figure 8.** Correlation plot of CsA concentrations by TOF-MS methods vs. HPLC. The correlation coefficient was determined to be 0.988 for the TOF-SIMS method and 0.986 for the MALDI/TOF-MS method.

**Figure 9.** CsA mono-hydroxylated metabolite concentrations determined by HPLC and both TOF-MS methods. Since all mono-hydroxylated metabolites of CsA are isobaric, the TOF-MS methods report total mono-hydroxylated CsA concentrations and HPLC reports each specific metabolite individually.
both TOF mass spectrometers. Note that in each of the four samples plotted in Fig. 9, the total concentrations of hydroxylated metabolites agree with each other within experimental error.

The results of quantifying total mono-hydroxylated metabolites determined by HPLC and both TOF-MS are listed in Table 7. As previously mentioned, sample no. 10 did not produce a quality chromatogram. In addition, blood sample no. 7 did not resolve the mono-hydroxylated species; therefore, peak areas could not be ascertained. A correlation plot of each TOF-MS method vs. HPLC of the remaining CsA metabolite determinations is shown in Fig. 10. The correlation coefficient ($r_0$) was determined to be 0.997 with the %RSD of the slope found to be 2.379% for the TOF-SIMS method, with $r_0 = 0.991$ and %RSD$_{slop e} = 4.075$

| Transplant sample no. | HPLC* (ng ml$^{-1}$) | TOF-SIMS (ng ml$^{-1}$) | MALDI/TOF-MS (ng ml$^{-1}$) |
|------------------------|-----------------------|--------------------------|-----------------------------|
| 1                      | <50                   | 31                       | 29                          |
| 2                      | 1947                  | 1938                     | 1615                        |
| 3                      | 752                   | 690                      | 494                         |
| 4                      | 856                   | 749                      | 677                         |
| 5                      | <50                   | 113                      | 143                         |
| 6                      | <50                   | 57                       | 56                          |
| 7                      | Did not resolve metabolites | 168                    | 207                        |
| 8                      | <50                   | 0                        | 82                          |
| 9                      | 551                   | 806                      | 550                         |
| 10                     | Poor chromatogram     | 8                        | 17                          |

* Summation of all mono-hydroxylated CsA species.

Application of TOF-SIMS and MALDI/TOF-MS in conjunction with data analysis techniques has allowed the simultaneous quantification of CsA and its major metabolite, AM1, in standard samples. This was accomplished using only one standard curve and has proven to be very rapid when compared to HPLC methodology. To resolve interferences, data analysis techniques have been used with both TOF-SIMS and MALDI/TOF-MS methods. The accuracy in determining CsA concentrations was not compromised by the presence of AM1 by either method; however, the use of data analysis techniques has allowed AM1 quantification within ~13% for the TOF-SIMS method and ~25% for the MALDI/TOF-MS. These results clearly indicate that by using a simple mathematical approach to resolve interferences in TOF-MS data, quantitative information can be obtained. Rapid establishment of the pharmacokinetics of immunosuppressive agents and characterization of the metabolism is possible with TOF-SIMS and MALDI/TOF-MS.

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QUANTIFICATION OF CYCLOSPORIN A AND METABOLITES

REFERENCES

1. R. J. Ptachinski, G. Burckart and R. Venkataramanan, J. Clin. Pharmcol. 26, 358 (1986).
2. R. J. Ptachinski, R. Venkataramanan and G. J. Burckart, Clin. Pharmacokinet. 23, 2732 (1996).
3. G. J. Burckart, A. Jain, W. Diven, R. Venkataramanan and T. E. Starzl, Transplant. Proc. 22 (3), 1319 (1990).
4. J. H. McBride, S. S. Kim, D. O. Rodgerson, A. F. Reyes and M. K. Ota, Clin. Chem. 38 (11), 2300 (1992).
5. J. C. K. Loo, K. D. Gallicano, I. J. McGilvery, N. Beaudoin and S. L. Jindal, Clin. Biochem. 24, 49 (1991).
6. V. F. J. Quensiaux, Clin. Biochem. 24, 37 (1991).
7. S. C. Lee, A. M. Brudzinski, J. L. Yasminneh, N. J. Johanson, I. A. Ferber, L. K. Mass, P. Y. Wong, P. A. Keown and J. W. Orf, Clin. Biochem. 24, 43 (1991).
8. P. P. Wang, E. Simpson, V. Meucci, M. Morrison, S. Lunetta, M. Zajac and R. Boecckx, Clin. Biochem. 24, 55 (1991).
9. P. P. Wang, E. Simpson, V. Meucci, M. Morrison, S. Lunetta, M. Zajac and R. Boecckx, Transplant. Proc. 22 (3), 1186 (1990).
10. L. J. Dusci, L. P. Hackett, G. M. Chiswell and K. F. Ilett, Therapeutic Drug Monitoring 14, 327 (1992).
11. P. Y. Wong, A. V. Mee, J. Glenn and P. A. Keown, Anal. Chem. 66 (14), 2362 (1994).
12. B. Hagenhoff, R. Kock, M. Deime, Benninghoven and H.-J. Bauch, in Secondary Ion Mass Spectrometry: Proceedings of the Eighth International Conference (SIMS VIII), ed. by A. Benninghoven, K. T. F. Janssen, J. Tumpner and H. W. Werner, p. 831. John Wiley & Sons, Chichester (1992).
13. B. Hagenhoff, R. Kock, E. Niehuis, A. Benninghoven, C. Wunschke and H. Musche, in Secondary Ion Mass Spectrometry: Proceedings of the Seventh International Conference (SIMS VII), ed. by Benninghoven, K. T. F. Janssen, J. Tumpner and H. W. Werner, p. 371. John Wiley & Sons, Chichester (1990).
14. D. C. Muddiman, A. J. Nicola, A. Proctor and D. M. Hercules, submitted to Appl. Spectrosc.
15. A. I. Gusev, W. R. Wilkinson, A. Proctor and D. M. Hercules, Anal. Chem. 67 (5), 1034 (1995).
16. C. P. Wang, N. R. Hartman, R. Venkataramanan, I. Jardine, F. T. Lin, J. E. Knapp, T. E. Starzl and G. T. Burckart, Drug Metab. Disip. 17, 292 (1989).
17. Abbott Laboratories, One Abbott Park Road, Abbott Park, IL 60064, USA.
18. E. Niehuis, in Secondary-ion Mass Spectroscopy, ed. by A. Benninghoven, p. 299. Wiley-Interscience, Chichester (1989).
19. E. Niehuis, T. Heller, H. Feld and A. Benninghoven, J. Vac. Sci. Technol. 45 (4), 1243 (1987).
20. E. Niehuis, P. N. T. van Veltzen, J. Lub, T. Heller and A. Benninghoven, Surf. Interface Anal. 14, 135 (1989).
21. GOOGLY Software, Copyright 1994 Andrew Proctor, All rights reserved.
22. A. I. Gusev, W. R. Wilkinson, A. Proctor and D. M. Hercules, Appl. Spectrosc. 47 (8), 1091 (1993).
23. P. J. Gemperline, S. E. Boyette and K. Tyndall, Appl. Spectrosc. 41, 454 (1987).
24. D. C. Muddiman, A. I. Gusev, L. B. Martin and D. M. Hercules, Fresnius’ J. Anal. Chem. in press.