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A Quantitative Study of *in vitro* Hepatic Metabolism of Tacrolimus (FK506) Using Secondary Ion and Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

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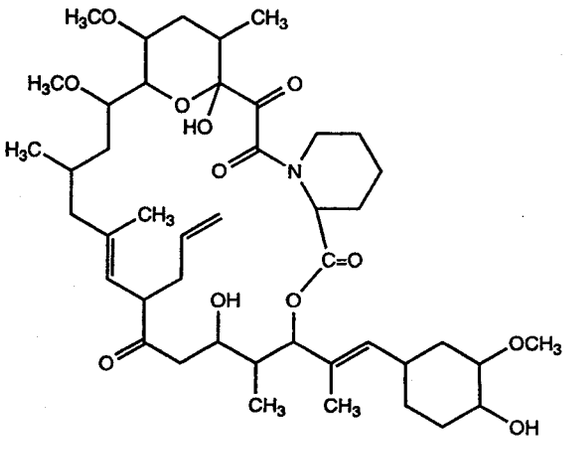
The identification and simultaneous quantification of Tacrolimus and its hepatic metabolites in baboons has been achieved using matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry and static secondary-ion mass spectrometry (TOF-SIMS). Little fragmentation, high sensitivity and tolerance to contamination are the major advantages of these methods, allowing facile identification and quantification of metabolites produced *in vitro* with minor analyte isolation. Based on the MALDI and TOF-SIMS results, seven metabolites have been identified: de-methylated, di de-methylated, hydroxylated, di hydroxylated, de-methylated hydroxylated, dihydrodiol, and di de-methylated hydroxylated. The concentrations of the parent drug and its major metabolites (e.g. de-methylated, di de-methylated) were measured using Rapamycin as an internal standard. The time course of Tacrolimus and its major metabolites as a function of incubation time was calculated. Good correlation between SIMS and MALDI results was obtained.

Tacrolimus (TAC) is a neutral lipophilic macrolide of known structure, shown in Fig. 1, isolated from the fermentation broth of a strain of *Streptomyces tsukubaensis*, and is a recently approved immunosuppressive agent, used efficiently in preventing the rejection of transplanted organs.[1-3] TAC is metabolized primarily in the liver by the cytochrome P450 IIIA enzyme subfamily, and yields several metabolites including demethylated TAC, hydroxylated and demethylated-hydroxylated TAC, and dihydrodiol TAC.[4-9] Some TAC metabolites show immunosuppressive effects;[6, 9, 10] therefore, quantification of TAC and determination of the kinetics of metabolite production are important[4, 5] for optimal therapy of transplant patients.

NMR and various mass spectrometric methods, including chemical ionization (CI), fast-atom bombardment (FAB), and liquid secondary-ion mass spectrometry (LSIMS), have been used[4-9] for the identification of TAC metabolites. However, these mass spectral techniques exhibit considerable fragmentation, which can result in an overestimation of metabolite concentrations (e.g. loss of the methyl group would be misinterpreted as a higher concentration of mono demethylated species) and poor sensitivity (i.e. the ion intensity is dispersed over several peaks). Furthermore, low molar absorptivity limits the use of high-performance liquid chromatography (HPLC) for metabolite separation and purification. To compensate for these problems, we applied two relatively new mass spectrometric techniques, matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry and static TOF secondary ion mass spectrometry (TOF-SIMS),[11, 12] to identify and quantify TAC metabolites. Both methods induce soft ionization, and have been proven to be quantitative techniques.[13-17] Recently, MALDI and TOF-SIMS have been applied successfully for the simultaneous quantitative analysis of the immunosuppressive drug cyclosporin A (CsA) and its major metabolite, monohydroxylated CsA (AM1), in the whole blood of organ transplant patients.[16] Good correlation was obtained between HPLC, which is the specific method for CsA analysis, and TOF-SIMS (r_o=0.988) and MALDI (r_o=0.986) results. In the present study, we report the identification and quantification of TAC metabolites generated in hepatic microsomes obtained from baboon livers. Baboons are used experimentally in xenotransplantation of liver and exhibit similar TAC metabolism to humans.[18] This report is also the first on the use of MALDI and TOF-SIMS



FK 506 [MW=803.5]

Figure 1. Molecular structure of Tacrolimus ($C_{44}H_{69}NO_{12}$).

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for identification and quantitative analysis of TAC metabolites. The investigation of *in vitro* systems not only allows an assessment of the preferred metabolic pathways, but the data can also be extrapolated to *in vivo* systems; the latter is true because the *in vitro* systems mimic *in vivo* systems, albeit on a compressed time-scale.

EXPERIMENTAL

Tacrolimus microsomal incubation

The baboon liver microsomes were prepared by differential centrifugation. Protein and cytochrome P450 concentrations were determined according to standard procedures.[19,20] Microsomal preparations (2 mg/mL) were incubated for 10, 20, 30, 45, and 60 min at 37 °C with TAC (Fujisawa Pharmaceutical, Japan) in the presence of 20 mM nicotinamide adenine dinucleotide phospate, reduced form (NADPH). The reaction was stopped by addition of 5 M HCl and rapid freezing. Portions (100–200 μL) of these incubates were extracted with diethyl ether and the ether was evaporated under a nitrogen stream. The residues were reconstituted in 150 μL of ethanol+water (1:1, v/v) before analysis. Rapamycin ($C_{51}H_{79}NO_{13}$) (Wyeth-Ayerst, USA) was used as a surrogate internal standard (IS).

Instrumentation used was a TOF-SIMS III (Ion-Tof GmbH, Münster, Germany) and a modified LAMMA 1000 (Leybold-Heraeus GmbH, Köln, Germany) MALDI TOFMS. The instruments and analytical protocols used for quantification have been described previously.[12,16,21,22]

Sample preparation for SIMS analysis involved deposition of a 1–2 μL aliquot of incubate on a previously etched high purity (99.9985%) silver substrate.[16] The multicomponent MALDI matrix used in this study was 2,5-dihydroxybenzoic acid (DHB) (20 mg/mL)+fucose (2 mg/mL) dissolved in 1:9 ethanol+water. Matrix (10 μL) was mixed with each incubate sample in a volume ratio of 1:1.[21,22] A 2 μL aliquot of this mixture was deposited on a polished stainless steel substrate and dried in high purity nitrogen.

RESULTS AND DISCUSSION

Metabolite identification

MALDI and SIMS spectra of the incubation mixtures of TAC (0, 30, and 45 min) are shown in Fig. 2. Extensive metabolism was detected by both methods. The mass differences between the peaks due to major metabolites and the parent-drug were found to be 14 u and 28 u, indicating that these are mono and di de-methylated species, respectively. The mass assignments of TAC and the metabolite peak positions in the MALDI and TOF-SIMS spectra are shown in Table 1. The major peaks in the MALDI spectra are due to Na- and K-cationization, which cause some interference with the hydroxylated Na-cationized metabolite and K-cationized TAC. Attempts to avoid this problem by adding a sodium salt did not give a sufficient increase of the Na-cationized peak compared to the K-cationized species because of the high potassium concentration in the incubates and the use of simple ether extraction. However, the relatively high intensities of the K-cationized peaks make it possible to use them for metabolite identification.

Further confirmation of metabolite identification was obtained using TOF-SIMS. This technique exhibits a different ionization mechanism for lipophilic materials deposited on silver, which are usually observed as silver-cationized species, see Fig. 2, and therefore provides complementary results. MALDI and SIMS gave similar results, based on which seven metabolites have been identified; they are listed, from major to minor abundance (signal intensity), in the caption of Fig. 2.

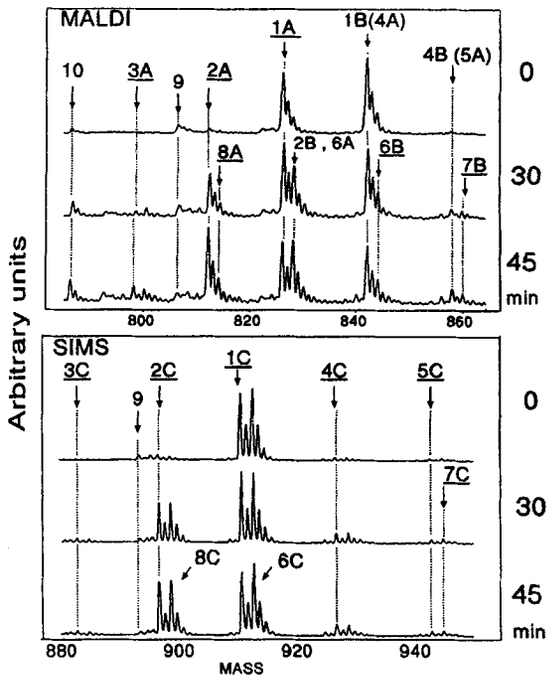

Figure 2. Positive MALDI and SIMS mass spectra of TAC incubates at 0, 30 and 45 min. 1: TAC; 2: de-methylated; 3: di de-methylated; 4: hydroxylated; 5: di hydroxylated; 6: de-methylated hydroxylated; 7: dihydrodiol; 8: di de-methylated hydroxylated; 9: TAC impurity; and 10: TAC, loss of water. A: sodium; B: potassium; and C: silver cationization. Peaks used for metabolite identification and quantification are underlined.

TAC and metabolite quantification

Quantification of TAC was carried out using rapamycin as the IS for SIMS and MALDI. Because, mass spectrometric methods cannot resolve metabolism occurring at different substituent positions, all isomeric forms are quantified together.

Initial attempts to use cyclosporin A (CsA) as an IS resulted in poor linearity of the standard curve.[23] CsA is routinely used as an IS in HPLC measurements, but its chemical properties differ from those of TAC. Although the

Table 1. Mass assignments of TAC and its metabolites in MALDI and SIMS spectra

		Peak position with cations (u)		
		MALDI		SIMS
#	Compound	Na^+	K^+	$^{107}Ag^+$
1	TAC	826	842	910
2	de-methylated	812	828	896
3	di de-methylated	798	814	882
4	hydroxylated	842	858	926
5	di hydroxylated	858	874	942
6	de-methylated hydroxylated	828	844	912
7	dihydrodiol	860	876	944
8	di de-methylated hydroxylated	814	830	898

use of an IS with chemical properties that differ from those of the analyte has been demonstrated for MALDI quantification, this use requires a constant (analyte+IS)/matrix molar ratio over the quantification range and stable instrumental conditions.[22] In the case of TAC, analysis was further complicated by the necessity to simultaneously quantify TAC and its metabolites in the presence of high contaminant concentrations. Since isotopically labelled TAC compounds are not available, rapamycin was used as an IS. Rapamycin has a chemical structure and ionization efficiency similar to that of TAC, and hence gave better results than CsA. Sodium and silver ^{107}Ag-cationized peaks were used to measure intensities in MALDI and SIMS experiments, respectively. The standard curves obtained for MALDI and SIMS are shown in Fig. 3. The relative standard deviations (RSD) of the TAC standard curve slopes, obtained by MALDI and SIMS, with rapamycin as an IS, were 1.3–2.5%, and the correlation coefficients (r_0) were 0.998–0.999.

The second step for quantification was to determine the relative ionization efficiencies (RIE) for TAC *versus* its metabolites (i.e. the metabolite response *vs.* that of the parent drug). Actually, the term RIE includes relative desorption/ionization and detector registration efficiencies. It is known that even small structural changes can lead to significant variations of RIE. For example, the RIE for the major CsA hydroxylated metabolite (AM1) *vs.* that of parent drug was found to be 4.45 for SIMS and 1.45 for MALDI.[16] However, the purity of the CsA metabolite used was unknown. In the case of TAC, pure metabolites are not currently available so that their RIEs could not be measured directly. It is however, possible to determine whether they are the same or differ. To determine whether the RIE values differ, the intensities of TAC peaks were summed along with the intensities of the metabolite peaks (*total intensity*) for each point of the time course for each method. Each sum was normalized to the rapamycin intensity. Figure 4 shows the dependance of the *total intensity*/rapamycin ratio as a function of incubation time.

It was found that the *total intensity*/rapamycin ratio was

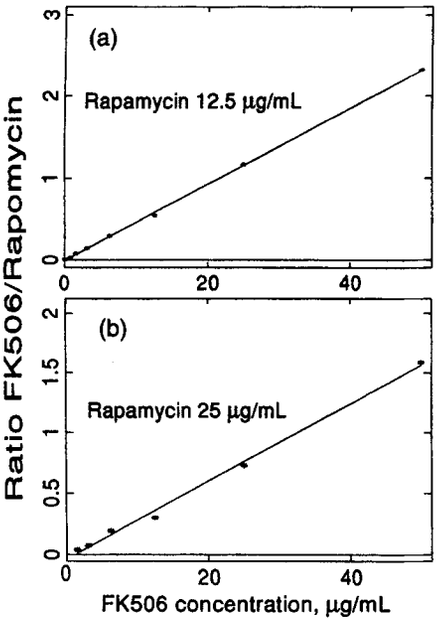

Figure 3. Standard curves obtained for analysis of TAC using (a) MALDI and (b) SIMS. Rapamycin was used as an internal standard.

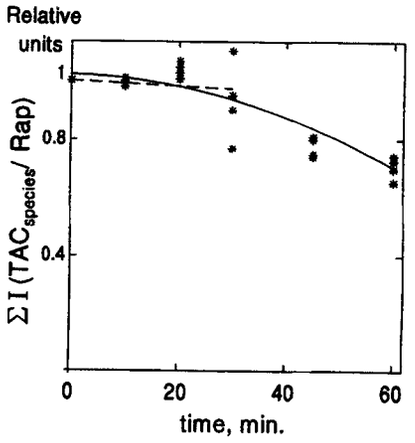

Figure 4. Dependence of the *total intensity*/rapamycin ratio as a function of incubation time. The ratio was normalized to 1.00 at t=0 min. – – –, the data were fitted to a linear function; ——, the data (0–60 min) were fitted to a quadratic function.

constant for 0–30 min with a small decrease at 45 and 60 min. Thus, the sum of total TAC and metabolite concentrations is also constant for 0–30 min and can be fitted using the linear function shown in Fig. 4. The equation of the curve was: $y=1-0.0008x$, where y is the normalized ratio and x is concentration in µg/mL. Strong metabolite peaks observed for 30 min (see Fig. 2) and the slope of the linearly fitted curve (nearly horizontal) indicate that the RIEs for TAC and its metabolites are essentially the same.

A small decrease of the *total intensity*/rapamycin ratio between 45 and 60 min can be explained by TAC decomposition. In this case, the dependence of ratio *vs.* time (45–60 min) is non-linear and was fitted by a second order polynomial approximation (quadratic function). Using the t statistics (t-test), it was found that both fits have correlation coefficients above the critical value at the 95% confidence limit. This supports the hypothesis that the RIEs of TAC and its metabolites are the same.

An important consideration is to estimate the precision of metabolite quantification. The overall precision of metabolite measurement can be estimated based on the RSD of slope of the linearly fitted curve decrease over 0–30 min.[15] The estimated precision of metabolite quantification was found to be *ca.* 20% (at the 95% confidence limit). However, variations of RIE for minor metabolites could be misinterpreted because their influence on the calculated ratios is small. Based on the slope value of the linearly fitted curve (decrease over 30 min), it was found that the hypothesis should be valid down to concentration of 2 µg/mL. This limits metabolite quantification to the two major metabolites: de-methylated and di de-methylated TAC.

Since the RIE for TAC and its major metabolites were found to be the same, the standard curve obtained for TAC can also be used for metabolite quantification. Figure 5 shows the time course of TAC, and the de-methylated and di de-methylated metabolites as a function of incubation time. Inspection of Fig. 5 indicates that the major metabolite, de-methylated TAC, is produced rapidly, compared to the other metabolites. Both mass spectral methods gave similar results; the concentrations of TAC, and of its demethylated and di de-methylated metabolites, measured by MALDI and SIMS, showed good correlation, r_0=0.913, 0.903 and 0.850, respectively, all of which are significant at the 95% confidence limit. This also confirms the validity of the hypothesis of equal RIEs for TAC and its major metabolites.

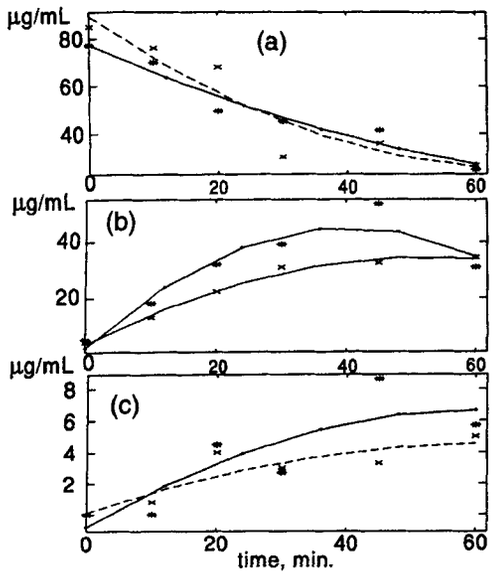

Figure 5. Concentration of (a) TAC, (b) mono and (c) di de-methylated metabolites as a function of incubation times using: ×--- SIMS and *—MALDI. The data were fitted to a quadratic function.

However, the decrease of the correlation coefficient may indicate that this hypothesis is not valid for minor metabolites.

CONCLUSIONS

MALDI and TOF-SIMS have been applied for identification and simultaneous quantificaton of Tacrolimus and its *in vitro* hepatic metabolites. Extensive metabolism was detected by MALDI and SIMS. Some interferences in MALDI spectra were avoided by use of Na- and K-cationized peaks for metabolite identification. TOF-SIMS produced similar results to MALDI and confirmed metabolite identification. Based on the MALDI and TOF-SIMS results, seven metabolites have been identified.

TAC and its two major metabolites (de-methylated and di de-methylated) have been quantified using rapamycin as an internal standard. Comparison of the *total intensities* of identified metabolites and TAC as a function of incubation time proved that they have similar RIEs; the TAC standard curve can be used for major metabolite quantification. The time course of TAC and its major metabolites as a function of incubation time was calculated. Good correlation between SIMS and MALDI results was obtained.

Thus, MALDI and SIMS were found to be the powerful tools for the qualitative and quantitative analysis of *in vitro* metabolism of TAC, demonstating that they are valuable techniques for *in vitro* and *in vivo* metabolic studies.

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