Tacrolimus (FK506) metabolite patterns in blood from liver and kidney transplant patients

ANN-KATRIN GONSCIOR, UWE CHRISTIANS, MICHAEL WINKLER, ANNETTE LINCK, JANA BAUMANN, and KARL-FRIEDRICH SEWING

The metabolite patterns of tacrolimus in blood were evaluated in 41 kidney and liver graft recipients. Trough concentrations of tacrolimus and its metabolites were measured by HPLC-mass spectrometry and microparticle enzyme immunoassay in parallel. A statistically significant correlation between results of both assays was observed for kidney and liver transplant patients \( r = 0.77, P < 0.001 \) and \( r = 0.71, P < 0.001 \), respectively. The main metabolites in blood were demethyl, demethyldihydroxy, didemethyl, didemethyldihydroxy, and hydroxy tacrolimus. These metabolites added up to 42\% (range 0–145\%) of the tacrolimus concentration in liver transplant patients and to 44.8\% (range 16–152\%) in kidney transplant patients. During episodes of impaired liver function, concentrations of tacrolimus and its metabolites were increased compared with normal liver function, indicating accumulation of metabolites, in particular second-generation metabolites such as didemethyl and didemethyldihydroxy tacrolimus. Stepwise regression analysis including tacrolimus, its metabolites, and liver function parameters suggested a model including serum activities of γ-glutamyltransferase, alkaline phosphatase, and alanine aminotransferase as predictors for increased concentrations of demethyl tacrolimus, didemethyl tacrolimus, and the parent drug.

INDEXING TERMS: high-performance liquid chromatography • mass spectrometry • metabolism • organ transplantation • therapeutic drug monitoring • microparticle enzyme immunoassay • macrolides • immunosuppressants

Tacrolimus (Prograf®, Fujisawa Pharmaceutical Co., Osaka, Japan) is a macrolide with potent immunosuppressive properties. It was introduced for primary and rescue immunosuppression in patients after solid organ transplantation and in immunological diseases [1–3]. In clinical trials, tacrolimus proved to be at least comparable with cyclosporine in preventing acute and chronic rejection in liver and kidney transplant patients [4–6]. Its clinical use is associated with the risk of nephrotoxicity, neurotoxicity, hypertension, and diabetogenic effects [7–10].

Tacrolimus is primarily metabolized by enzymes of the cytochrome P-450 3A subfamily [11–13]. The main reactions tacrolimus undergoes during metabolism are demethylation and (or) hydroxylation [14]. To date, nine metabolites have been isolated by HPLC [14–16]. First-generation metabolites are formed by metabolism of tacrolimus in one position, and second-generation metabolites by further metabolism of first-generation metabolites.

Immunologic assays have been developed for therapeutic drug monitoring of tacrolimus [17–19]. Because these methods do not allow discrimination between parent drug and metabolites, little is known about the metabolite patterns in various transplant patients and the possible influence of liver and kidney function on metabolite concentrations in blood. To evaluate possible toxic effects of the metabolites and the clinical relevance of their immunosuppressive activity [20, 21], further assessment of metabolite patterns in transplant recipients is required. Therefore, we aimed here to study the influence of kidney and liver function on tacrolimus metabolite patterns in blood by using a specific HPLC-mass spectrometry (MS) assay.

**Materials and Methods**

**PATIENTS**

Forty-one patients who underwent either orthotopic liver transplantation \( n = 21 \) or kidney transplantation \( n = 20 \) were included in the study. The mean age was 43 years, ranging from...
17 to 65 years. The mean body weight was 68 kg (range 48 to 96 kg). One patient received liver and kidney transplants and was allocated to the liver recipient group. The liver recipient group included 15 male and 6 female, the kidney recipient group 15 male and 5 female patients.

**IMMUNOSUPPRESSIVE REGIMEN**

Tacrolimus was administered orally every 12 h. Doses were adjusted on the basis of trough blood tacrolimus concentrations as measured by microparticle enzyme immunoassay (MEIA) and according to the clinical status. The target tacrolimus trough blood concentrations during a stable long-term course for liver transplant patients were in the range of 3–8 µg/L and 5–15 µg/L for kidney transplant patients [19]. In the kidney recipient group, 18 patients received prednisolone (0.21 ± 0.09 mg kg⁻¹) and 16 patients azathioprine (1.57 ± 0.5 mg kg⁻¹) as additional immunosuppressants in the early posttransplant period. In the liver recipient group, 11 patients received prednisolone (0.1 ± 0.04 mg kg⁻¹).

**BLOOD SAMPLE COLLECTION AND ANALYSIS**

Trough blood samples were collected into EDTA-containing tubes and kept at 4 °C until analysis. Tacrolimus and its metabolites were measured by an HPLC-MS assay as described earlier [22]. The method was based on solid–liquid extraction from blood combined with mass spectrometric detection of the drug and its metabolites after isocratic elution by HPLC with methanol/water as mobile phase. The concentrations were determined with 32-O-acetyltacrolimus as internal standard. The lower limit of quantification was 0.2 µg/L. The interassay variability during the study was 14.5% at 5 µg/L, the accuracy was 6% at 5 µg/L, and the calibration curve was linear from 0.2 to 100 µg/L. An aliquot of each blood sample was measured in parallel by MEIA with the IMx® analyzer (Abbott, Abbott Park, IL) according to the manufacturer’s instructions. Liver function [serum bilirubin, activities of alanine aminotransferase (ALT; EC 2.6.1.2), aspartate aminotransferase (ASAT; EC 2.6.1.1), γ-glutamyltransferase (GGT; EC 2.3.2.2), alkaline phosphatase (AP; EC 3.1.3.1), cholinesterase (CHS; EC 3.1.1.7), glutamate dehydrogenase (GLDH; EC 1.4.1.2)] and kidney function (serum urea and creatinine concentrations), hematocrit (HCT), and hemoglobin (HB) were measured in parallel in the Institut für Klinische Chemie (Medizinische Hochschule Hannover, Hannover, Germany) with standard biochemical methods.

**DATA ANALYSIS**

All patients, ranging from the day of transplantation to 3 years after transplantation, were included in the study, thus reflecting several phases of transplant function. Since the observation period lasted for 4 months, multiple data sets were obtained from single patients.

To evaluate the correlation between HPLC-MS assay and the MEIA, trough blood samples from various kidney (n = 87) and liver (n = 84) transplant patients were analyzed in parallel, and correlation and regression analysis were calculated (procedures Corr and Reg, SAS statistics package, version 6.05; SAS Institute, Cary, NC).

**ANALYSIS OF TACROLIMUS METABOLITE PATTERNS AT DIFFERENT STAGES AFTER KIDNEY OR LIVER TRANSPLANTATION**

To analyze the tacrolimus metabolite patterns early after transplantation and later, data sets were allocated to two groups: (a) group “day 0–14”, in which data were obtained during the early postoperative period (from the day of transplantation until the 14th day after transplantation), and (b) group “day >14”, in which data were recorded after the 14th posttransplantation day.

Liver and kidney function as well as blood parameters of all patients and the respective groups are shown in Table 1. Means ± SD of tacrolimus and metabolite concentrations were calculated by using the univariate procedure (SAS). Because data were not normally distributed, the two groups were compared by using Wilcoxon’s test (Nparlway, SAS).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Liver graft recipients</th>
<th>Kidney graft recipients</th>
</tr>
</thead>
<tbody>
<tr>
<td>All data sets</td>
<td>Data set day 0–14</td>
<td>Data set day &gt;14</td>
</tr>
<tr>
<td>ALAT, U/L</td>
<td>127 ± 292</td>
<td>356 ± 495</td>
</tr>
<tr>
<td>ASAT, U/L</td>
<td>95 ± 279</td>
<td>284 ± 494</td>
</tr>
<tr>
<td>AP, U/L</td>
<td>476 ± 396</td>
<td>188 ± 80.1</td>
</tr>
<tr>
<td>GGT, U/L</td>
<td>183 ± 163</td>
<td>99.2 ± 45.9</td>
</tr>
<tr>
<td>GLDH, U/L</td>
<td>120 ± 669</td>
<td>394 ± 1229</td>
</tr>
<tr>
<td>CHS, ku/L</td>
<td>2.66 ± 1.6</td>
<td>2.35 ± 1.17</td>
</tr>
<tr>
<td>Bilirubin, µmol/L</td>
<td>138 ± 139</td>
<td>249 ± 188</td>
</tr>
<tr>
<td>HB, g/L</td>
<td>103 ± 18.1</td>
<td>107 ± 14</td>
</tr>
<tr>
<td>HCT, %</td>
<td>29.3 ± 6.34</td>
<td>31 ± 4.8</td>
</tr>
<tr>
<td>Creatinine, µmol/L</td>
<td>158 ± 79.1</td>
<td>198 ± 107</td>
</tr>
<tr>
<td>Urea, mmol/L</td>
<td>28.4 ± 32.6</td>
<td>20.7 ± 8.5</td>
</tr>
</tbody>
</table>

Data sets "day 0–14" and "day >14" were compared by Wilcoxon’s test. Significance level: ***, P < 0.001; **, P < 0.01; *, P < 0.05.

All enzymes, bilirubin, creatinine, and urea were measured in serum.
BLOOD CONCENTRATIONS OF TACROLIMUS AND ITS METABOLITES AND LIVER OR KIDNEY FUNCTION

The association between liver function and tacrolimus metabolite patterns was analyzed by stepwise regression (procedure Stepwise, SAS). Concentrations of didemethyl tacrolimus (second-generation metabolite), demethyl tacrolimus (first-generation metabolite), and tacrolimus were selected as dependent variables. All liver function parameters and the serum concentrations of urea and creatinine were selected as independent variables. The significance level for entry into the model was \( P < 0.15 \). The Stepwise regression procedure was performed for all data sets of liver recipients and separately for data sets between 0 and 14 days and >14 days posttransplantation. The correlation between tacrolimus metabolite concentrations and parameters for cholestasis such as serum bilirubin and serum activities of AP and GGT was analyzed (procedures Corr and Reg, SAS).

Data sets of liver graft recipients with good or impaired liver function were selected by using the following selection criteria, which were based upon the 50% quantiles after calculating distribution statistics, including all data sets of liver graft recipients. The liver function parameters were chosen according to the results obtained by stepwise regression analysis: impaired liver function = \( AP \geq 277 \text{ U/L}^{-1}, \text{ALAT} \geq 40 \text{ U/L}^{-1}, \) and \( \text{GGT} \geq 127 \text{ U/L}^{-1} \); good liver function = \( AP < 277 \text{ U/L}^{-1}, \text{ALAT} < 40 \text{ U/L}^{-1}, \) and \( \text{GGT} < 127 \text{ U/L}^{-1} \). The patient who also received a kidney graft was excluded from this analysis. Biochemical parameters of each group are shown in Table 2. Concentrations of tacrolimus and its metabolites were compared by using Wilcoxon's test (SAS).

Accordingly, the influence of kidney function on tacrolimus metabolism was studied by selecting patients with good or impaired kidney function from the kidney recipients group. The following selection criteria based on the 50% quantiles were chosen: impaired kidney function (n = 33) = creatinine \( \geq 198 \text{ \mu mol/L}^{-1} \) and urea \( \geq 15.2 \text{ mmol/L}^{-1} \), and normal kidney function (n = 39) = creatinine <198 \text{ \mu mol/L}^{-1} \) and urea <15.2 \text{ mmol/L}^{-1}.

Doses and liver function were not significantly different in both groups of kidney graft patients.

Results

The main metabolites of tacrolimus found in blood were demethyl tacrolimus, demethylhydroxy tacrolimus, didemethyl tacrolimus, didemethylhydroxy tacrolimus, and hydroxy tacrolimus. These metabolites added up to 42% of the tacrolimus blood concentration (ranging from 0% to 145%) in the liver recipient group and to 44.8% (ranging from 16% to 152%) in the kidney recipients. A difference between both groups became more evident during the early posttransplantation period ("day 0-14"). In the liver recipient group the sum of metabolites represented 35% of the tacrolimus blood concentration (hydroxy tacrolimus could not be detected in this subgroup) compared with 45.2% in the kidney recipient group (\( P < 0.001 \)). However, in single liver transplant patients an increase of trough blood concentrations of metabolites has been observed during episodes of severe cholestasis (vide infra).

A significant correlation between tacrolimus concentrations measured by HPLC-MS (\( \alpha \)) and MEIA (\( \gamma \)) is evident in kidney (\( r = 0.77 \)) and liver graft recipients (\( r = 0.71 \)). The following equations were calculated by linear regression analysis: For liver graft recipients (Fig. 1A), \( y = 0.64 (\pm 0.07)x + 2.65 (\pm 0.78)\mu g/L, S_{p} = 0.38, P < 0.001; \) for kidney graft recipients (Fig. 1B), \( y = 0.65 (\pm 0.06)x + 3.75 (\pm 0.84)\mu g/L, S_{p} = 0.27, P < 0.001.

A linear regression analysis between tacrolimus concentrations and doses revealed that, despite higher doses in kidney transplant patients, tacrolimus concentrations were relatively lower than in liver transplant patients. The following regression equations were calculated: For liver graft recipients, tacrolimus concentration (\( \mu g/L \) = 0.25 \cdot \) dose (mg/day) + 7.7; for kidney graft recipients, tacrolimus concentration (\( \mu g/L \) = 0.14 \cdot \) dose (mg/day) + 10.6.

The metabolite patterns in liver and kidney graft recipients during the early and late postoperative periods are shown in Fig. 2. In liver transplant patients, all detected metabolites and tacrolimus were higher during the late postoperative period ("day >14") compared with earlier. In kidney transplant patients, only tacrolimus and demethylhydroxy tacrolimus concentrations were higher during the postoperative period.

The doses required to obtain tacrolimus blood trough concentrations in the therapeutic range were significantly higher in kidney than in liver graft patients (Fig. 2. Fig. 3 shows the correlation of didemethyl tacrolimus with bilirubin (\( r = 0.18, P = n.s. \)), AP (\( r = 0.54 \)), and GGT (\( r = 0.46 \)) in liver transplant patients. The following equations were calculated by linear regression analysis (mean \pm SD): didemethyl tacrolimus (\( \mu g/L \) = 0.01 (\( \pm 0.0002 \)) \cdot AP (U/L) + 0.074 (\( \pm 0.13 \)), \( S_{p} = 0.21, P < 0.001; \) didemethyl tacrolimus (\( \mu g/L \) = 0.002 (\( \pm 0.0006 \)) \cdot GGT (U/L) + 0.175 (\( \pm 0.135 \)), \( S_{p} = 0.23, P < 0.001. \) There was no correlation between liver function and concentrations of other tacrolimus metabolites.

Similar results were obtained with a stepwise regression analysis including all data sets of liver graft recipients. For concentrations of didemethyl tacrolimus and demethyl tacrolimus as dependent variables, a model was suggested including the

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Table 2. Biochemical parameters of liver graft recipients with impaired liver function (ILF) and with good liver function (GLF) (mean ± SD).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ILF (n = 12)</th>
<th>GLF (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALAT, U/L</td>
<td>88.8 ± 63</td>
<td>24.2** ± 6.9</td>
</tr>
<tr>
<td>ASAT, U/L</td>
<td>46 ± 15.9</td>
<td>13.4** ± 7.2</td>
</tr>
<tr>
<td>AP, U/L</td>
<td>1005 ± 384</td>
<td>174** ± 60.9</td>
</tr>
<tr>
<td>GGT, U/L</td>
<td>422 ± 184</td>
<td>48.3** ± 26</td>
</tr>
<tr>
<td>GLDH, U/L</td>
<td>26.2 ± 20.3</td>
<td>7.6** ± 14.9</td>
</tr>
<tr>
<td>CHS, km/L</td>
<td>2.08 ± 1.2</td>
<td>3.66 ± 2.1</td>
</tr>
<tr>
<td>Bilirubin, \mu mol/L</td>
<td>202 ± 121</td>
<td>58.7** ± 69.9</td>
</tr>
<tr>
<td>HB, g/L</td>
<td>100 ± 20</td>
<td>110 ± 21.4</td>
</tr>
<tr>
<td>HCT, %</td>
<td>28.6 ± 5.5</td>
<td>31.2 ± 6.2</td>
</tr>
<tr>
<td>Creatinine, \mu mol/L</td>
<td>174 ± 72.4</td>
<td>121 ± 55.2</td>
</tr>
<tr>
<td>Urea, mmol/L</td>
<td>27.4 ± 11.2</td>
<td>37.7 ± 65.4</td>
</tr>
</tbody>
</table>

Significance level: ** \( P < 0.01 \); * \( P < 0.05 \) (Wilcoxon's test).
liver function parameters AP and ALAT as independent predictors. AP was suggested as the most appropriate parameter for tacrolimus concentrations in blood (as measured by MEIA). When only data from 0–14 days posttransplantation were included, GGT was suggested as predictor for concentrations of tacrolimus, didemethyl tacrolimus, and demethyl tacrolimus as dependent variables. There was no association with AP and ALAT in the early period after transplantation (except for demethyl tacrolimus and ALAT, $P < 0.001$).

The metabolite and tacrolimus concentrations in liver graft recipients with impaired and good liver function are shown in Fig. 4. Tacrolimus doses were significantly reduced in patients with cholestasis ($P < 0.05$). Tacrolimus blood concentrations as measured by HPLC-MS and MEIA were not significantly different in both groups, although a tendency towards higher tacrolimus and metabolite concentrations during episodes of impaired liver function was obvious (Fig. 4). Concentrations of didemethyl tacrolimus and didemethylhydroxy tacrolimus were significantly higher in patients with impaired liver function. The increase in metabolite concentrations did not parallel increased tacrolimus concentrations, but was associated with a shift of the metabolite pattern towards second-generation metabolites during episodes of impaired liver function. During periods of normal hepatic function, didemethyl and didemethylhydroxy tacrolimus both averaged 5.5% of the total metabolite fraction, whereas during episodes of liver dysfunction these concentrations increased to 17.5% and 10.4%, respectively. Relative concentrations of demethyl tacrolimus decreased from 41.4% to 32.1% and concentrations of demethylhydroxy tacrolimus from 46.6% to 38.6% during episodes of impaired liver function. The mean concentrations of all metabolites added up to 50% (range 0–104%) of the tacrolimus concentration and were significantly higher than in data sets representing good liver function ($P < 0.05$, Wilcoxon’s test). However, this ratio was remarkably higher in individual patients with severe cholestasis. Fig. 5 shows an HPLC-MS ion chromatogram of a liver transplant patient during cholestasis. In this case, the metabolite fraction adds up to ~74% of the tacrolimus concentration.

Patients with impaired kidney function in the kidney recipient group showed significantly reduced tacrolimus concentrations ($P < 0.005$) compared with patients with good kidney function. There were no differences in dose and metabolite concentrations.

**Discussion**

We show in this study that tacrolimus metabolite concentrations and the metabolite patterns depend on the type of graft, time after transplantation, and liver function. The doses required to maintain tacrolimus trough blood concentrations in the therapeutic range were approximately two times higher in kidney than in liver graft patients. There are several possible explanations: The blood circulation through the liver is likely to be impaired in liver graft patients. Therefore, the extraction of tacrolimus from blood and elimination by the liver might be lower than in kidney graft patients. Furthermore, the activity of cytochrome P-450 enzymes may be lower in liver graft patients, resulting from damage of the liver graft during storage and
transport before transplantation and impairment of blood circulation during the early posttransplantation period. Additionally, kidney graft patients received two times higher steroid doses than liver graft patients. Steroids are well-known inducers of cytochrome P-450 3A enzymes [23] and might result in a lower bioavailability [24] and faster elimination of tacrolimus in kidney than in liver graft patients.

For data sets obtained from liver graft patients during the early posttransplant period, an association between serum activity of GGT and concentrations of demethyl, didemethyl, and tacrolimus was observed, reflecting the impact of cholestasis [25] on tacrolimus elimination and blood concentrations of tacrolimus and its metabolites during the early posttransplant period. Later, the influence of liver function on tacrolimus elimination seems to be better reflected by changes in serum activities of AP and ALAT. Stepwise regression analysis included ALAT, AP, and GGT in a model to predict impaired tacrolimus elimination. In liver graft patients receiving cyclosporine, only bilirubin strictly parallels the trough blood concentrations of second-generation metabolites [26]. The correlation with the serum activity of the cytosolic enzyme ALAT rather than with indicators for cholestasis such as bilirubin implied that the degree of liver dysfunction resulting in increased tacrolimus and metabolite concentrations has to be more severe than that resulting in impaired cyclosporine elimination.

Cholestasis leads to increased trough blood concentrations of tacrolimus metabolites as well as to an alteration of the metabolite pattern, which is caused by an overproportional increase of second-generation metabolites such as didemethyl and didemethylhydroxy tacrolimus. This result indicates that the formation of tacrolimus during cholestasis was impaired to a lesser extent than excretion of the metabolites into the bile duct. When passage through the biliary membrane is hampered, the metabolites usually excreted into bile accumulate in the hepatocyte and enter the blood. Since second-generation metabolites reach several times higher concentrations in bile than first-generation metabolites (unpublished data), this is a possible explanation for the observed shift of the tacrolimus metabolite patterns in blood. Additionally, first-generation metabolites, which usually undergo biliary elimination, remain in the hepatocyte in higher concentrations than normal, facilitating further metabolism to second-generation metabolites.

Comparable alterations of the metabolite patterns have been reported for cyclosporine in bone marrow transplant recipients with graft-vs-host disease affecting the liver and kidney graft patients with cholestasis [27, 28]. In the present study, the increased metabolite concentrations during cholestasis did not parallel increased tacrolimus concentrations, which was the result of blood concentration-guided dosing regimen.

The antibody upon which the MEIA is based cross-reacts with several tacrolimus metabolites [20, 21, 29], especially 15-O- and 31-O-demethyl tacrolimus. All other metabolites cross-react
Fig. 3. Correlation of didemethyl tacrolimus with liver function parameters [(A) bilirubin, (B) GGT, and (C) AP] in liver transplant patients.

<10%. In the present study, as in a pharmacokinetic study [30], HPLC-MS and MEIA resulted in equal tacrolimus concentrations. This might be explained by the low concentrations of 15-O- and 31-O-demethyl tacrolimus compared with metabolites that cross-react with the antibody to a small extent, such as 13-O-demethyl tacrolimus. The correlation coefficients for tacrolimus concentrations measured with MEIA and HPLC-MS in the present study are similar to those ($r = 0.73$) recently described by Winkler et al. [31] for HPLC-MS and a commercial ELISA that is based upon the same antibody as the MEIA. Even in data sets during cholestasis, the mean tacrolimus concentrations measured by MEIA equaled those measured by HPLC-MS. Second-generation metabolites such as didemethyl and didemethylhydroxy tacrolimus, the concentrations of which were increased during cholestasis, cross-react with the MEIA antibody <1% [21, 29]. It is therefore unlikely that these metabolites significantly influence the result of MEIA measurement [21]. Therapeutic drug monitoring exclusively on the basis of MEIA bears the potential risk of accumulation of tacrolimus metabolites in blood without notice. This is especially important
since there seems to be a higher risk of tacrolimus toxicity in patients with liver dysfunction [32].

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References