Sensitive and specific quantification of sirolimus (rapamycin) and its metabolites in blood of kidney graft recipients by HPLC/electrospray–mass spectrometry

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Sirolimus (rapamycin) has a macrolide structure and is under clinical investigation as an immunosuppressant after organ transplantation. An HPLC/mass spectrometry assay to quantify sirolimus in blood was developed. 28-O-Acetyl sirolimus was used as internal standard. Blood samples were extracted with C18 columns. The extracts were injected into an HPLC system and isocratically eluted with methanol/1% formic acid (90/10 by vol) from a 150 × 4 mm C18 analytical column. The HPLC system was connected to a triple-stage quadrupole mass spectrometer with an electrospray interface and positive ions were detected. The limit of quantification in 1 mL of blood was 0.25 µg/L and the calibration curve in blood was linear up to 250 µg/L. The recovery from blood was 88 ± 26% and interassay variation at 1 µg/L was 19% and at 15 µg/L 9.3%. Hydroxy, dihydroxy, demethyl, and didemethyl sirolimus as well as sirolimus were detected in blood of kidney graft patients.

INDEXING TERMS: therapeutic drug monitoring • organ transplantation • macrolides

Sirolimus (rapamycin, AY-22,989; Wyeth-Ayerst, Princeton, NJ) is a 31-membered macrolide lactone C51H79N13O13 [1–3] with a molecular mass of 913.6 Da (Fig. 1). In solution, sirolimus forms two conformational trans-, cis-isomers with a ratio of 4:1 (chloroform) due to hindered rotation around the pipelic acid amide bond [3, 4]. Because of its triene structure, sirolimus has ultraviolet (UV) absorption maxima at 288, 276, and 266 nm.6 It is sparingly soluble in water, aliphatic hydrocarbons, and diethyl ether, whereas it is soluble in alcohols, halogenated hydrocarbons, and dimethyl sulfoxide [1, 5, 6]. Sirolimus is unstable in solution and degrades in plasma and low- and neutral-pH buffers at 37 °C, with a half-life of <10 h [6, 7]. The structures of the degradation products have recently been characterized [8].

Sirolimus is isolated from Streptomyces hygroscopicus [5, 9] and was originally developed as an antifungal [6, 10, 11] and anticancer drug [6, 12]. Interest has also focused on its immunosuppressive activity [13]. Although it shares structural homology with the immunosuppressant tacrolimus and binds to the same intracellular binding protein in lymphocytes [14, 15], it inhibits S6p70-kinase and therefore has a mechanism of immunosuppressive action distinct from that of tacrolimus [6, 16]. Sirolimus was found to prolong graft survival of different transplants in several species alone [6, 17–19] or in combination with other immunosuppressants [6, 20, 21]. In animal models its spectrum of toxic effects is different from that of cyclosporine or tacrolimus [6, 22], comprising impairment of glucose homeostasis, stomach ulceration, weight loss, and thrombocytopenia [6, 17, 21], although no nephrotoxicity has been detected [18, 21]. Sirolimus is under clinical investigation as an immunosuppressant after kidney transplantation.

Sirolimus is metabolized by cytochrome P-450 3A to at least six metabolites [23, 24]. During incubation with human liver and small intestinal microsomes, sirolimus was hydroxylated and (or) demethylated, and the structure of 39-O-demethyl sirolimus was identified [24]. In bile of sirolimus-treated rats, >16 hydroxyl-
ated and (or) demethylated metabolites were detected by HPLC/electrospray–mass spectrometry (ESI-MS) [25].

HPLC assays have been described by several authors [24, 26–29]. Most of these methods do not allow quantification of sirolimus metabolites [26–29] and have other serious drawbacks: The method described by Sattler et al. [23] does not report any specifications, Napoli and Kahan [26] did not show that their method is able to quantify sirolimus in biological samples, and Yatscoff et al. [27] used demethyl sirolimus as internal standard, which is a potential sirolimus metabolite [24] and is present in blood of patients in concentrations equal to that of sirolimus (vide infra). The only HPLC/UV assay described so far that is used for therapeutic drug monitoring of sirolimus-treated patients is that described by Napoli and Kahan [29]. This assay does not allow quantification of metabolites and its sensitivity is limited to 2 μg/L. Because of its high immunosuppressive potency [6, 16, 30], a sensitive and specific assay for sirolimus and its metabolites that detects concentrations <1 μg/L in tissue and biological fluids is required to evaluate its pharmacokinetics [6].

**Materials and Methods**

**INSTRUMENTS AND CHEMICALS**

The following HPLC/ESI-MS/MS system was used: A TSQ 700 mass spectrometer with electrospray fast-flow interface (Finnigan MAT, Bremen, Germany) was combined with an L6200 HPLC pump and an L7200 autosampler (Merck-Hitachi, Darmstadt, Germany). A 3.9 × 150 mm Resolve® analytical C_{18} column with 5-μm particle size (Waters Millipore, Milford, MA) was used as analytical column and all solvents for sample extraction or HPLC/MS analysis were purchased from Merck (Darmstadt, Germany) and were of HPLC quality. For semi-preparative isolation of sirolimus metabolites as well as the internal standard, an HP 1084B chromatograph (Hewlett Packard, Waldbronn, Germany) in combination with a 655A-40 injector and a D-2000 integrator (both Merck-Hitachi) and a 10 × 250 mm column filled with Nucleosil® C_{8} reversed-phase material (Macherey-Nagel, Düren, Germany) with 7-μm particle size were used. Acetic anhydride for synthesis of the internal standard was of reagent grade and purchased from Merck. Samples were extracted on 3-mL C_{18} columns (Recipe, Munich, Germany) with a Speed-Mate® extraction bench (Zinsser Analytic, Frankfurt, Germany). Sirolimus was a kind gift from S.N. Schgal (Wyeth-Ayerst, Princeton, NJ). Blood samples were taken from four stable kidney graft recipients (avg. 89 days after transplantation, ranging from 25 to 155 days) receiving oral sirolimus therapy who were included in the phase II clinical trial at the Texas Medical Center (Houston, TX). The patients received a mean sirolimus dose of 1.35 mg/day (range: 0.9–1.9 mg/day). All patients received cyclosporine and prednisolone as additional immunosuppressants. Samples were anticoagulated with EDTA. The collection of blood samples for measurement of sirolimus concentrations was part of the study protocol, which was in accordance with the Declaration of Helsinki of 1975 and its revisions and was approved by the local ethics committee.

**STORAGE OF PATIENTS’ SAMPLES AND STOCK SOLUTIONS**

Because the instability of sirolimus is one of the critical issues of its analytics, all patients’ samples were placed on ice immediately, deep frozen at −40 °C as soon as possible, and were mailed on dry ice. Stock solutions of sirolimus and its internal standard 28-O-acetyl sirolimus were dissolved in methanol and stored at −80 °C. Under these conditions, patients' samples and stock solutions were stable for at least 6 months. After 6 months the concentration of sirolimus was 88 (11)% [mean (SD), n = 8] of the initial concentration. After a second freeze/thaw cycle, the concentration of sirolimus was significantly reduced or below the detection limit. Stability of sirolimus and its metabolites during HPLC/MS analysis was checked as follows: Sirolimus was metabolized by human liver microsomes as described previously [23] and sirolimus and its metabolites were quantified by HPLC-UV [23]. To 1 mL of blood, 100 μL of the extracted microsomes containing sirolimus and its metabolites as well as 250 ng of internal standard were added. The blood samples were extracted as described below. The extracts were pooled and placed in the autosampler of the HPLC/MS system. One hundred microliters was injected and analyzed five times immediately after extraction and after 1, 3, 5, and 7 days as described below. The results of days 1–7 were expressed in percent of the concentrations measured right after extraction (=100%).

![Fig. 1. Structure of sirolimus and its metabolic pathways.](http://example.com/fig1.png)
SYNTHESIS AND PURIFICATION OF THE INTERNAL STANDARD
The internal standard 28-O-acetyl sirolimus and two other mono- as well as one diacetylated sirolimus derivative were synthesized as follows: 12.85 mg of sirolimus was dissolved in 13 mL (137.5 mmol) of acetic acid anhydride, resulting in a final concentration of 14 mmol/L, and incubated for 3 h at 55 °C. The mixture was dried under a stream of nitrogen and the residue was dissolved in 2 mL of acetonitrile/sulfuric acid, pH 3, (75/25 by vol) and injected into a semipreparative HPLC system. The acetylated sirolimus derivatives were separated on a 250 × 10 mm C8 7-μm column by using the following acetonitrile/sulfuric acid (pH 3) gradient: analysis time 0 min, 400 mL/L acetonitrile; analysis time 38 min, 700 mL/L acetonitrile; analysis time 45 min, 800 mL/L acetonitrile. The flow was set to 4.5 mL/min, the column temperature to 35 °C, and the UV detection wavelength to 276 nm. 28-O-Acetyl sirolimus was eluted from the HPLC column with a retention time of 32 min (Fig. 2). Fractions were manually collected and extracted with equal volumes of dichloromethane. The organic phase was dried over MgSO4 and evaporated under vacuum. Structures and purity of the internal standard were confirmed by ESI-MS/MS and collision-activated dissociation (CAD). For CAD, argon was used as collision gas with a collision energy of 49 eV. Structural identification of the internal standard was based upon analysis of the fragmentation pattern shown in Figs. 3 and 4 and described in refs. 31 and 32.

GENERATION AND PURIFICATION OF SIROLIMUS METABOLITES
Sirolimus metabolites were generated as previously described [24]. Human liver microsomes were isolated by using standard centrifugation techniques [33] and the protein concentration adjusted to 3 g/L with 0.1 mmol/L phosphate buffer. Sirolimus was dissolved in acetonitrile/sulfuric acid (pH 3, 75/25 by vol) and incubated with 1 mL of the microsomal suspension and 0.5 mL of an NADPH-producing system (2 mmol/L EDTA, 10 mmol/L MgCl2, 0.84 mmol/L NADP, 18 mmol/L isocitric acid, and 667 U/L isocitrate dehydrogenase dissolved in 0.1 mmol/L phosphate buffer, pH 7.4) for 30 min at 37 °C. The reaction was stopped by addition of 0.5 mL of acetonitrile. The samples were centrifuged at 2500g for 2 min and the supernatant drawn through glass extraction columns (Kranich, Göttingen, Germany) filled with C8 LiChroprep® (Merck) of 25–40-μm particle size and previously washed with 3 mL of acetonitrile and 3 mL of sulfuric acid (pH 3). The samples were washed on the columns with 3 mL of methanol/sulfuric acid (pH 3, 50/50 by vol) and 1 mL of hexane and eluted with 1.5 mL of dichloromethane. The dichloromethane eluates were pooled, evaporated at 40 °C under a stream of nitrogen, and the residues dissolved in 1.5 mL of acetonitrile/sulfuric acid (pH 3, 75/25 by vol). The sample was washed with the same volume of hexane and then injected into the HPLC system. Sirolimus and its metabolites were eluted from a 250 × 4 mm C8, 3-μm Nucleosil column with the following gradient: analysis time 0 min, 470 mL/L acetonitrile; 7 min, 470 mL/L acetonitrile; 20 min, 500 mL/L acetonitrile; 40 min, 550 mL/L acetonitrile; 45 min, 610 mL/L acetonitrile. The flow was set to 0.7 mL/min, the oven temperature to 35 °C, and the detection wavelength to 276 nm. Fractions containing the metabolites were manually collected and the metabolites identified by HPLC/MS/MS as previously described [24] and as described for the internal standard above (key fragments of 39-O-demethyl sirolimus: see Fig. 4). Purity of the isolated metabolites was >95% as estimated by HPLC/UV [24]. Furthermore, the metabolites were used to compare the signal intensities of the different sirolimus

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**Fig. 2. Isolation of the internal standard 28-O-acetyl sirolimus and other acetylated sirolimus derivatives by semipreparative HPLC.**

Chromatogram of 28-O-acetyl sirolimus during semipreparative isolation by HPLC. HPLC conditions: 250 × 10 mm C8 7-μm column, acetonitrile/sulfuric acid (pH 3) gradient: analysis time 0 min, 500 mL/L acetonitrile; analysis time 20 min, 550 mL/L acetonitrile; analysis time 35 min, 650 mL/L acetonitrile; analysis time 40 min, 750 mL/L acetonitrile. Flow 4.5 mL/min, column temperature 35 °C, UV detection wavelength 276 nm. A representative chromatogram is shown. Retention time (tR) 28.5 min, sirolimus (A, larger peak); tR 32.9 min, 28-O-acetyl sirolimus (B, larger peak); tR 34.9 min, 40-O-acetyl sirolimus (C, larger peak); tR 37.6 min, 28,40-Diacetyl sirolimus (D, larger peak). The smaller peaks behind the sirolimus and derivative peaks with retention times of 29.9 min, 33.8 min, 36.0 min, and 38.5 min, respectively, represent the separated rotamers.
derivatives and recoveries of single metabolites during extraction with those of sirolimus and the internal standard.

**EXTRACTION OF BLOOD SAMPLES**

To 1 mL of blood, 25 μL of the internal standard solution (1 mg/L in methanol) and for protein precipitation 2 mL of methanol/water saturated with zinc sulfate (70/30 by vol) were added. Samples were vortex-mixed for 20 s and centrifuged at 2000g for 2 min. The supernatants were drawn through C18 extraction columns that had previously been primed with 2 mL of acetonitrile and 2 mL of sulfuric acid (pH 3). The pressure was adjusted to −5 mmHg. The extraction columns were washed with 2 mL of sulfuric acid (pH 3) and dried by drawing air through the columns for 2 min. Sirolimus, its metabolites, and the internal standard were eluted with 400 μL of acetonitrile/1% formic acid (90/10 by vol). Two hundred microliters were transferred into an HPLC microval (Hewlett-Packard) and 100 μL of the extract was injected into the HPLC system.

**HPLC/ESI-MS**

Sirolimus, its metabolites, and the internal standard were eluted isocratically from the analytical column with methanol/1% formic acid (90/10 by vol) at a flow rate of 0.4 mL/min. The column temperature was 35 °C. The mass spectrometer was tuned with 0.5 g/L sirolimus dissolved in the mobile phase and delivered by a syringe pump at 5 μL/min, bypassing the HPLC column. The capillary temperature was 200 °C. The auxiliary gas pressure was 175 kPa and the gas was not heated. The sheath gas pressure was 175 kPa. The needle voltage was adjusted to 5.5 kV and the capillary exit voltage to −200 V. The mass spectrometer was run in the positive ion mode. The multiplier voltage was set to 1400 V and the conversion dynode to −15 kV. For single ion detection, the MS was focused on the [M+Na]+ of didemethyl sirolimus (908 atomic mass unit (amu)), demethyl sirolimus (922 amu), sirolimus (936 amu), hydroxy sirolimus (952 amu), dihydroxy sirolimus (968 amu), trihydroxy sirolimus (984 amu), as well as 28-O-acetyl sirolimus (978 amu). The resolution was set to 1000 (10%-valley definition) and a mass was scanned within 0.25 s.

Because sirolimus and its metabolites gave the same detector response after MS analysis as equal amounts of the internal standard, similar slope and intercept of the calibration curves, as well as equal recoveries (see below), concentrations of sirolimus and its metabolites were calculated as follows: sirolimus or metabolite (μg/L) = (areas sirolimus or metabolite/areas internal standard) · internal standard added (μg/L). Determination of recoveries, inter- and intraassay variation, calibration curves, and lower limit of quantification were based on blood collected from healthy volunteers, to which sirolimus or the purified metabolites dissolved in acetonitrile/sulfuric acid (pH 3, 7/3 by vol) were added. Calibration curves were made up of six data points (0, 1, 5, 10, 50, and 250 μg/L with n = 5). The concentration of sirolimus or its metabolites was calculated by using the internal standard and the formula described above. Data were analyzed by using linear regression analysis (REG procedure, SAS version 6.05; SAS Institute, Cary, NC). The accuracy was calculated as the deviation from the nominal concentration added to 1-mL blood samples of healthy volunteers and are given in percent of the nominal concentration. As for the calibration curve, concentrations were calculated with
the internal standard. For determination of the lower limit of quantification, samples containing the following concentrations were prepared: 0, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 µg/L. The lower limit of quantification was calculated as the percentage deviation from the nominal concentration obtained by inverse prediction from the standard regression curve. For acceptance as lower limit of quantification, the percentage deviation for a certain concentration had to be <25% for at least 80% of the samples.

To assess a potential interference with the sirolimus degradation products described by Wang et al. [8], these were produced by using the method described [8] with a few modifications: One milligram of sirolimus was dissolved in 100 µL of ethanol and incubated with 2 mL of 0.1 mol/L ammonium acetate (pH 8). The degradation products were separated on a 250 × 4 mm column filled with Hypersil C18 3-µm material. The flow rate was 0.7 mL/min and the same methanol/0.05 mol/L ammonium acetate gradient as in the original method [8] was run. The UV-detector wavelength was set to 276 nm and fractions were manually collected. Two hundred microliters of the collected fractions were injected in the HPLC/MS system used for quantification of sirolimus and its metabolites.

**Results**

The reaction of sirolimus with acetic acid anhydride yielded two mono- and one diacetylated derivatives, which were isolated by semipreparative HPLC (Fig. 2) and identified by ESI-MS (Figs. 3 and 4). Their structures were identified by ESI-MS/MS and CAD. In Fig. 3 representative CAD spectra of sirolimus and the internal standard 28-0-acetyl sirolimus are displayed. The structural assignment was based on high resolution and linked-scan fast-atom bombardment data of sirolimus recently published by Kiplinger [31] and Kiplinger and Guadillana [32]. These data could be applied as well to the analysis of the sodium adduct ions of sirolimus, its metabolites, and acetylation products by using ESI-MS/MS and CAD (Fig. 4). Thus, the two isomeric monoacetyl derivatives 28-0- and 40-O-acetyl sirolimus could be distinguished on the basis of their characteristic...
fragments at m/z 589 and 387, respectively (Fig. 4). As checked by HPLC/MS, the internal standard preparation was not contaminated with sirolimus. When the internal standard solution was added to blank blood samples, no material interfering with the quantification of sirolimus or its metabolites was detected.

The stability of sirolimus, the internal standard, and two metabolites are shown in Fig. 5. The concentrations measured at different days were compared by analysis of variance combined with Duncan grouping (GLM procedure, SAS version 6.05), which indicated significant differences (P < 0.0001 for sirolimus and all derivatives). Duncan grouping showed that the concentrations of sirolimus and 39-O-demethyl sirolimus at day 5 and that the concentrations of all sirolimus derivatives at day 7 were lower than those measured earlier. There was no significant difference when the concentrations of sirolimus, 28-O-acetyl sirolimus, and the metabolites measured on the same day were compared with each other (analysis of variance). When the concentrations were calculated by using the internal standard, analysis of variance showed that the concentrations were equal until day 5. Concentrations of sirolimus (P < 0.04) and 39-O-demethyl sirolimus (P < 0.03) measured at day 7 were significantly different from those during the first 5 days because of signals close to the detection limit and unreliable quantification. Thus, extracts containing sirolimus, the internal standard, and sirolimus metabolites stored at room temperature in an autosampler were stable for at least 3 days and allowed a reliable quantification until 5 days after sample preparation.

During the extraction procedure, 88.3 (26)% sirolimus [mean (SD), n = 10], 85 (14.7)% 39-O-demethyl sirolimus, 90.6 (23)% hydroxy sirolimus, 90.5 (25)% dihydroxy sirolimus, and 86.3 (16.2)% internal standard were recovered. The recoveries were not different from each other (analysis of variance). The detection of positive ions proved to be 10 times more sensitive than the detection of negative ions. In blood samples, sirolimus, its metabolites, and the internal standard were mainly detected as the sodium adduct [M+Na]+: The relative intensity of [M+H]+ was <5% of the sodium adduct. No fragments were detected at a nozzle-skimmer voltage >-250 V. The [M+Na]+ were detected with the highest sensitivity at -200 V. At a nozzle-skimmer voltage < -250 V, fragmentation into the two main fragments [24] and several smaller fragments (<m/z = 250) was induced. Injection of 1 ng of sirolimus resulted in a detector response of 4326 (745) areas [mean (SD), n = 6], of 1 ng of 39-O-demethyl sirolimus in 3978 (545) areas, of 1 ng of hydroxy sirolimus in 4193 (264) areas, and of 1 ng of the internal standard 28-O-acetyl sirolimus in 4006 (305) areas. The detector responses were equal (analysis of variance). At >500 mL/L methanol in the mobile phase, sensitivity of sirolimus detection was independent of the mobile phase composition. Sirolimus (retention time tR = 6.5 min after injection), its metabolites (tR = 3.8-5.4 min), and the internal standard (tR = 6.8 min) were eluted from the analytical column as different peaks. The absolute limit of detection defined as the signal-to-noise ratio of 3:1 after flow injection of sirolimus calibration solutions was 25 pg. The lower limit of quantification after extraction from 1 mL of blood, injection of 100 μL of the extract into the HPLC system, and elution from the analytical column was 250 pg for sirolimus, its metabolites 39-O-demethyl and hydroxy sirolimus, and the internal standard. The calibration curve of sirolimus in blood was linear from 0.25 to 250 μg/L, with y = 0.91 (0.03)x + 0.9 (1.8) [mean (SD); r = 0.98, P < 0.0001]. Regression analysis of the calibration curves of 39-O-demethyl, hydroxy, and 28-O-acetyl sirolimus gave the following respective results: y = 0.86 (0.04)x + 1.5 (0.8) (r = 0.97, P < 0.0001), y = 1.03 (0.09)x - 0.5 (1.3) (r = 0.98, P < 0.0001), and y = 0.95 (0.07)x - 1.1 (2.1) (r = 0.98, P < 0.0001). The accuracy was -2.5% (range -17-10%, n = 10), +6.9% (range -18-19%, n = 10), and -4% (range -22-7.2%, n = 6) for 1-mL blood samples to which 1, 5, and 250 ng of sirolimus were added, respectively. The inter- and intraassay variations at different sirolimus concentrations are listed in Table 1. The intraassay variation of 39-O-demethyl sirolimus was 10.3% [concentration 5.6 (0.6) μg/L, mean (SD), n = 6] and the interassay variation 11.8% [concentration 5.3 (0.6) μg/L, n = 6]; the intraassay variation of hydroxy sirolimus was 9.5% [concentration 4.9 (0.5) μg/L, n = 6] and the interassay variation 12.3% [concentration 4.7 (0.6) μg/L, n = 6]. The run time of a sample was 8 min and the sample turnover time was 6 min, since samples were injected 2

![Graph](image-url)

Fig. 5. In-process stability of sirolimus, its metabolites, and the internal standard 28-O-acetyl sirolimus.

Samples were prepared and analyzed as described in Materials and Methods. They were stored in the autosampler at room temperature. Concentrations were calculated with an external sirolimus calibration curve. The results of days 1-7 were expressed in percent of the concentrations measured right after extraction (=100%). Each data point is the mean (SD) of five subsequent injections.

| Table 1. Intra- and interassay variation of the measurement of sirolimus concentrations in blood by HPLC/ESI-MS. |
|---|---|---|---|---|---|
| Interassay | Intrassay | n | Mean (SD), μg/L | CV, % | n | Mean (SD), μg/L | CV, % |
| | | | | | | | | |
| 10 | 0.96 (0.14) | 15.4 | 10 | 1.1 (0.2) | 19.5 |
| 10 | 4.6 (0.45) | 9.9 | 10 | 4.7 (0.6) | 12.8 |
| 10 | 14.6 (0.9) | 5.9 | 7 | 14.8 (1.4) | 9.3 |
| 8 | 39.6 (4.5) | 11.4 | 8 | 41.3 (6) | 14 |
| 6 | 247.3 (28.8) | 11.6 | 6 | 241.7 (31) | 13 |
min before the end of the previous analysis. The HPLC/ESI-MS system was stable for at least 200 subsequent injections of blood sample extracts. No interference of sirolimus degradation products with the quantification of sirolimus or its metabolites was detected.

Sirolimus and its metabolites were quantified in blood of kidney graft patients. Typical ion chromatograms of patients' samples in blood are shown in Fig. 6. In blood at various times after sirolimus application comprising peak as well as trough concentrations, hydroxy (1.4–23 μg/L), dihydroxy (2.2–11 μg/L), demethyl (0–15 μg/L), and didemethyl (0–15 μg/L) sirolimus were found. Sirolimus concentrations ranged from 3 to 42 μg/L (n = 13). In trough blood samples, the metabolites accounted for 56 (9)% [mean (SD)] of all sirolimus derivatives measured with hydroxy sirolimus, reaching 71.8 (68)% of the sirolimus concentration, demethyl sirolimus 35 (42)% dihydroxy sirolimus 20 (20)% and didemethyl sirolimus 20 (31)%. The trough blood concentrations of sirolimus ranged from 3 to 26 μg/L (n = 5).

**Discussion**

HPLC/ESI-MS represents a simple and sensitive technique for the specific quantification of sirolimus and its metabolites in blood and probably in other biological fluids as well. Analysis of sirolimus is complicated by its instability and the formation of conformational rotamers. Its instability [6] required long-term storage of samples at −80 °C and the development of an extraction procedure that avoided thermally induced degradation of the sirolimus derivatives such as evaporation steps. In previously described extraction procedures [23, 24, 27], samples had to be washed to remove material interfering with UV detection before HPLC analysis. Such a step was unnecessary for MS analysis. The conformational rotamers of sirolimus can be incompletely separated on analytical HPLC columns leading to broad peaks [26]. Peak broadening was avoided by using the chromatographic conditions described by Napoli and Kahan [26]. The specific mass spectrometric detection allowed quantification of hydroxylated and (or) demethylated metabolites. The chromatographic method described was not able to discriminate

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**Fig. 6.** HPLC/ESI-MS ion chromatogram of sirolimus and its metabolites in five blood samples obtained from kidney graft patients.

HPLC: 3.9 x 150 mm analytical column filled with 3-μm C18 material, isocratic elution with methanol/1% acetic acid (90/10 by vol), flow 0.4 mL/min. To save time, the next sample was injected during the run of the previous sample. All ions were detected as sodium adducts [M+Na]+. (A) Total ion count, (B) 936.7 amu: sirolimus, (C) 978.7 amu: 26-O-acetyl sirolimus (internal standard), (D) 922.7 amu: demethyl sirolimus, (E) 952.7 amu: hydroxy sirolimus.
metabolites with the same modifications in different positions. The method was also not able to distinguish between the $^{13}$C-signal of hydroxylated and the $^{12}$C signal of demethylated and hydroxylated metabolites such as hydroxy sirolimus and dihydroxydemethyl sirolimus. Such metabolites can only be differentiated by using time-consuming gradient elution [23-25]. Since it was shown that in the HPLC/MS method described, sensitivity of sirolimus detection is independent of the composition of the methanol/water phase, it can be combined with gradient elution to increase specificity of metabolite detection. Only the metabolites 39-O-demethyl and hydroxy sirolimus were available for this study. Therefore, only these metabolites were included in the method validation. Additionally, didemethyl and dihydroxy sirolimus could be detected in blood of patients. Quantification of these metabolites had to be based on the assumption that their recoveries from blood, the calibration curves, and their ionization and detection in the MS was similar to that of sirolimus, the internal standard, and the other metabolites.

For quantification of the structurally related macrolide immunosuppressant tacrolimus and its metabolites, an MS method based on a particle beam interface and chemical ionization has been described [34, 35]. This technique could not be used for sirolimus because of its extensive fragmentation during ionization. The use of an internal standard was necessary to compensate for losses during extraction, in-process instability of sirolimus and its metabolites, transport of extracted samples, and for decreased sensitivity of mass spectrometric detection due to contamination, especially of the electrospray interface. Acetylated derivatives have been used for MS quantification of tacrolimus as well [34, 35]. They result in an m/z that, in contrast to demethyl sirolimus, which has also been used as an internal standard for an HPLC assay [27], can clearly be distinguished from sirolimus and its metabolites. Internal standards other than sirolimus derivatives such as tacrolimus or β-estradiol-3-methyl ether [29] seem to be unsuitable since their stability during storage and their in-process stability may be different from that of sirolimus.

In the present study, identification of the structures of the acetylated sirolimus derivatives and the metabolites was based on the fast-atom bombardment results described by Kiplinger and Guadliana [32]. High-energy single-collision CAD fragmentation observed in magnetic sector instruments [32] might differ from that observed in low-energy multiple-collision CAD fragmentation in a triple-stage quadrupole instrument as used in the present study. However, since fragmentation patterns corresponded to those observed by Kiplinger and Guadliana [32], it could be assumed that during low- as well as high-energy CAD, sirolimus and its derivatives were undergoing comparable fragmentation pathways.

The issue of whether to measure immunosuppressants in plasma or blood has been discussed for cyclosporine and tacrolimus [36-38]. Because of their temperature-, concentration-, and hematocrit-dependent distribution in blood and their high affinity for the cellular components, blood is currently the generally accepted matrix for these immunosuppressants. Since almost 95% of sirolimus in blood is found in erythrocytes, blood was recommended as the matrix for sirolimus as well [39] and therefore chosen as the matrix in this study.

Several metabolites isolated after in vitro metabolism of sirolimus [24] and detected in bile of sirolimus-treated rats [25] were present in the blood of kidney transplant patients. In contrast to tacrolimus, which accounts for >80% of all tacrolimus derivatives in the blood of patients with stable liver function, sirolimus metabolites add up to higher concentrations than the unchanged drug.

Sirolimus has a low oral bioavailability and a narrow therapeutic range in animal models [22]. Its elimination is very likely dependent on liver function, and drug-related side effects appear to be trough concentration related [22]. Furthermore, sirolimus is metabolized by enzymes of the cytochrome P-450 3A subfamily in the liver and small intestine [23, 24], with the potential risk of numerous interactions at this level with drugs usually administered after organ transplantation [40], resulting in modification of its trough blood concentrations. All this, as well as experience with tacrolimus and cyclosporine, requires the clarification of whether or not blood concentration-guided dose adjustments and regular therapeutic drug monitoring of sirolimus will be necessary. HPLC/UV has been proposed as the method of choice [22], mainly on the basis of animal studies [22, 41]; the trough and peak blood concentrations in patients reported in our study are lower than those reported in animal studies [42]. Furthermore, the metabolites have generally lower concentrations [6, 42], which allows even lower doses resulting in lower blood concentrations. In this regard, modification of this HPLC/ESI-MS will allow quantification of cyclosporine and sirolimus as well as their metabolites within one run. In comparison with HPLC, HPLC/ESI-MS requires less elaborate extraction of blood samples than HPLC/UV and allows quantification of sirolimus and its metabolites within 6 min. In comparison, an HPLC/UV assay that is used for therapeutic drug monitoring of sirolimus [29] takes >30 min to quantify sirolimus and does not determine its metabolites. HPLC/ESI-MS allows analysis of an urgent sample within <1 h as well as analysis of large numbers of samples for pharmacokinetic studies and therapeutic drug monitoring.

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References