

Quantification of Sirolimus by Liquid Chromatography-Tandem Mass Spectrometry Using On-Line Solid-Phase Extraction

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Quantification of the new immunosuppressant sirolimus (syn. rapamycin; Rapamune®) in whole blood by chromatography is essential for its clinical use since no immunoassay is available although monitoring is mandatory. Here we report on a rapid and convenient liquid chromatography (LC)-tandem mass spectrometry method and describe our practical experience with its routine use.

Whole blood samples were hemolyzed and deproteinized using an equal volume (150 µl) of a mixture of methanol/zinc sulfate solution containing the internal standard desmethoxy-rapamycin. After centrifugation, the clear supernatants were submitted to an on-line solid-phase extraction procedure using the polymeric Waters Oasis HLB® material, with elution of the extracts onto the analytical column in the back-flush mode by column switching. For analytical chromatography a RP-C18 column was used with 90/10 methanol/2 mM ammonium acetate as the mobile phase. A 1:10 split was used for the transfer to the mass spectrometer, a Micromass Quattro LC-tandem mass spectrometry system equipped with a Z-spray® source and used in the positive electrospray ionization mode. The following transitions were recorded: sirolimus, 931>864 m/z, and desmethoxy-rapamycin (I.S.), 901>834 m/z. The analytical running time was 5 min, including on-line extraction.

The method has a linear calibration curve ($r > 0.99$; range 1.6–50 µg/l) and is rugged and precise with monthly CVs <7% at a sirolimus concentration of 13.1 µg/l in routine use; the instrumentation proved to be reliable and required minimal maintenance. Clin Chem Lab Med 2002; 40(1):40–45

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Abbreviations: LC-tandem MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring; RP, reversed phase; SPE, solid-phase extraction.

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Introduction

Sirolimus (syn. rapamycin; Rapamune®, Wyeth-Ayerst, Princeton, NJ, USA) is a newly introduced immunosuppressant consisting of a 3-membered macrolide ring. It modulates the immune response by inhibiting the activity of a regulatory protein critical to the coordination of events required for cells to move from the G1 to the S phase of the cell cycle; this is in contrast to cyclosporin and tacrolimus which achieve their effects by inhibiting calcineurin and reducing interleukin-2 expression. An essential advantage of sirolimus compared to calcineurin inhibitors, especially in renal transplant patients, is the absence of nephrotoxicity (1). Additionally, sirolimus exerts antiproliferative effects and may reduce the incidence of graft vessel disease (2–4).

The necessity for the therapeutic monitoring of sirolimus has meanwhile become evident and is recommended for all patients. Enteral resorption of the drug is very poor and shows important inter-individual variation. Sirolimus is extensively metabolized by the hepatic cytochrome P₄₅₀ system, with probably little contribution of its metabolites to immunosuppressive effects (5). A large number of common drugs may increase (e.g. cyclosporin, calcium antagonists, macrolide antibiotics, prokinetika, H₂-receptor antagonists) or decrease (e.g. anticonvulsants, tuberculostatica) sirolimus whole blood concentrations. While whole blood trough concentration below approximately 6 µg/l is associated with an increased risk of organ rejection after kidney transplantation (in combination with cyclosporin), trough concentrations above approximately 15 µg/l are associated with myelosuppression (predominantly thrombocytopenia) and hypercholesterolemia (6, 7). Pharmacological characteristics of sirolimus have been extensively reviewed (7–10).

During phase II and III clinical trials, an automated immunoassay manufactured by Abbott (Abbott Laboratories, Abbott Park, IL, USA) has been used for sirolimus monitoring (11); this assay, however, is not available any more. In consequence, the clinical introduction of sirolimus – which has been meanwhile licensed for the treatment of renal transplant patients and investigated in phase III clinical studies in heart and liver transplant patients – at present critically depends on the availability of suitable analytical methods for therapeutic drug monitoring.

Several HPLC methods for the determination of sirolimus, employing either UV-detection or mass spectrometry, have been published previously. Due to the poor UV-absorption of sirolimus, quantification using UV-detection is cumbersome and highly efficient sample clean-up is necessary (12–15). Liquid chromatogra-

phy with single-stage detection by mass spectrometry (LC-MS) overcomes the poor UV-absorption of the sirolimus molecule but requires also efficient chromatographic separation due to the limited specificity of this detection principle (16–20). In contrast, liquid chromatography-tandem mass spectrometry (LC-tandem MS) (syn. LC/MS/MS) offers a far higher specificity, so that chromatography can be substantially simplified, resulting in short analytical run times. However, an efficient sample clean-up procedure is required in order to prevent malfunctions of the LC-tandem MS system due to clogging of capillaries and ion suppression effects. Previously described methods for the quantification of sirolimus in whole blood by LC-tandem MS employ off-line solid phase extraction (11, 21, 22), solvent extraction (23) or simple on-line solid phase extraction procedure as reported by Volosov *et al.* (24). Here we report long-term experience with an optimized LC-tandem MS method with on-line solid-phase extraction; in contrast to the latter method, a separate extraction column containing a copolymer material is used and elution of the analyte from the extraction column onto the analytical column is done in a back-flush mode.

Materials and Methods

Chemicals

Sirolimus (mw 914.187) and 32-desmethoxy-rapamycin (AY-24668-1; mw 884.161) used as the internal standard were a gift from Wyeth-Ayerst Research (Princeton, NJ, USA). Stock solutions were prepared in methanol (50 mg/l).

Zinc sulfate heptahydrate (Merck, Darmstadt, Germany) was dissolved in water to obtain a zinc sulfate concentration of 50 g/l; a precipitation solution was made of methanol and this zinc sulfate solution by mixing them in 4/1 proportion (v/v). Ammonium acetate was from Merck (Darmstadt, Germany); a 200 mM stock solution was made in water for preparation of the mobile phase. Methanol and water were of HPLC grade (Baker, Deventer, The Netherlands).

Instruments

The Waters Alliance 2690 HPLC separation module (Waters, Milford, USA) consisting of an autosampler, a gradient pump and a column oven was used, coupled with a split of approximately 1:10 to a Micromass Quattro LC-tandem MS system equipped with a Z-spray[®] source (Micromass, Manchester, UK). An additional isocratic HPLC pump was from Merck Hitachi (model L-6000; Merck Darmstadt, Germany).

Standards and samples

The stock calibrator was prepared by spiking a drug-free hemolyzed whole blood pool with the sirolimus stock solutions to a concentration of 50 µg/l; after careful mixing and overnight equilibration additional working calibrators were made by serial dilution (25–12.5–6.25–3.1–1.6 µg/l), frozen at –70 °C and used in each series together with a zero-calibrator.

Sample preparation

A semi-automated two-step sample preparation protocol consisting of a manual deproteinization step and an automated (on-line) coupled-column solid phase extraction (SPE) procedure was applied.

In 2.0 ml polypropylene cups (Eppendorf, Hamburg, Germany) 150 µl of calibrators or carefully resuspended whole blood samples, were precipitated with methanol/zinc sulfate (50 g/l) 4/1 (v/v) containing desmethoxy-rapamycin (15 µg/l) as an internal standard. After vigorous vortex mixing the samples were centrifuged in a benchtop centrifuge at 15 000 *g* for 10 min; 150 µl of the clear supernatants were transferred into the HPLC vials and placed in the autosampler.

For on-line solid phase extraction the Waters Oasis HLB[®] column (50×1.0 mm; Waters, Milford, USA) was used together with a six-port high-pressure switching valve (Rheodyne, Rohnert Park, CA, USA) which was installed in the Waters 2690 separation module and controlled by the Micromass Masslynx 3.4 software.

The operating procedure for the HPLC-integrated (on-line) sample clean-up system consisted of three steps: first, 50 µl of deproteinized sample were injected into the system by the autosampler and transferred onto the Oasis HLB[®] extraction column. Here sirolimus was adsorbed, whereas potentially inter-

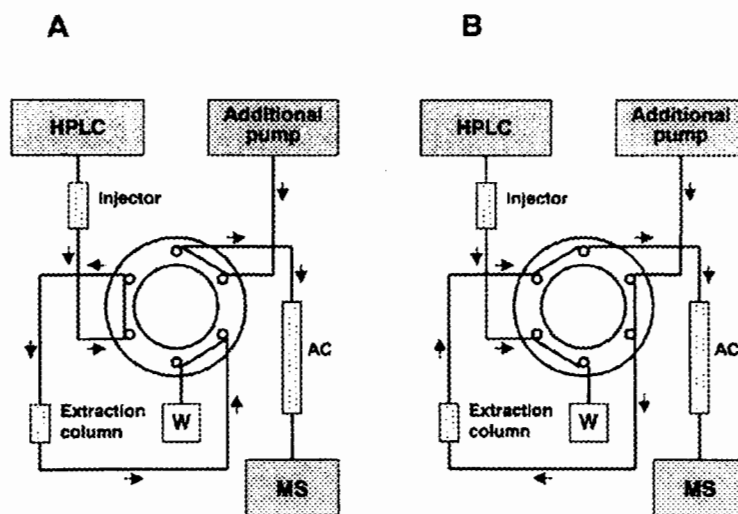


Figure 1 Column-switching scheme during the on-line extraction procedure (W, waste; AC, analytical column). A: Load-

ing of the sample onto the extraction column and washing; B: Elution from the extraction column onto the analytical column.

fering matrix compounds were washed directly into the waste by a mobile phase consisting of water/methanol, 95/5 (v/v) delivered at a flow rate of 4 ml/min for 1 min (Figure 1, valve position A). In parallel, the analytical column (Reprosil pur C18-AQ, 125x2 mm; 5 µm, Maisch, Ammerbuch, Germany) was equilibrated with methanol/2 mM ammonium acetate, 90/10 (v/v) with a flow rate of 0.5 ml/min. Following this on-line sample clean-up step, the six-port valve was switched to position B; the extract was now eluted in the back-flush mode from the Oasis HLB[®] column and transferred onto the analytical separation column (mobile phase: methanol/2 mM ammonium acetate, 90/10 (v/v); flow rate: 0.5 ml/min; time: 2 min). After the transfer of sirolimus and the internal standard was completed, the valve was switched back to position A and isocratic HPLC separation was performed under the same chromatographic conditions as described for the analyte transfer. Simultaneously, the Oasis HLB[®] extraction column was washed with pure methanol at a flow rate of 4 ml/min for 1 min and subsequently re-equilibrated with water-methanol 95/5 (v/v). Both the extraction and the analytical column were kept out at 35 °C in a column oven.

The retention time of sirolimus and the internal standard, respectively, was approximately 2.9 min, a complete analysis cycle including SPE and separation took 5 min.

Mass spectrometric conditions

Electrospray atmospheric pressure ionization in the positive mode was used; the source parameters were tuned to obtain the ammonium adduct ions of sirolimus and desmethoxy-rapamycin (931.5 m/z and 901.5 m/z), respectively; the following settings resulted in optimum ion yield: capillary voltage, 3.0 kV; cone voltage, 25 V; source temperature 80 °C; desolvation temperature 230 °C at a nitrogen flow of approximately 630 ml/min and a nebulizer gas flow of approximately 75 ml/min. The collision energy with argon as the collision gas was 20 V. Under these conditions an intense daughter ion of 864.5 m/z was obtained from sirolimus, and of 834.5 m/z from desmethoxy-rapamycin. Thus, the following ion transitions were used for multiple reaction monitoring (MRM) from minute 1 to 5 after sample injection: sirolimus 931.5>864.5 m/z, and desmethoxy-rapamycin 901.5>834.5 m/z. The dwell time in both MRM traces was 0.5 s, interchannel delay and interscan delay were 0.15 s.

For quantification, the response of calibrator and serum samples was calculated as the peak area ratio of the MRM trace of sirolimus and the internal standard, respectively (single smoothing with a window size of one scan; polynomial type: linear; fit weighting: 1/x).

Assay validation

To investigate the analytical recovery of the extraction procedure and possible ion-suppression effects, the standard solutions of each sirolimus and desmethoxy-rapamycin in water/methanol (90/10) at three concentration levels (5, 10, and 50 µg/l) were introduced as a bolus into a continuous flow of the mobile phase by the built-in high-pressure valve of the MS system; for comparison, whole blood calibrator samples, prepared by spiking to the same concentrations as the standard solutions (5, 10, and 50 µg/l), were submitted to the complete assay as ordinary samples using a splitless mode of introduction to the MS system. The experiments were carried out in duplicate. Mean peak areas of the respective MRM traces of the analytes were compared between the two settings (direct injection of standard solution vs. on-line extraction and HPLC of spiked whole blood samples) to calculate a recovery rate taking into account the dilution of the whole blood samples

during protein precipitation with methanol/zinc sulfate solution.

To study the linearity of the method, regression analysis of the six calibrator samples run in four series was carried out; the inaccuracy was determined for the calibrator samples by comparing the recalculated concentrations with weighted target concentrations.

To investigate the imprecision of the method, three whole blood pools were prepared from residual samples from patients treated with sirolimus, in a low, medium, and high concentration range, aliquoted and kept frozen at -70 °C. Analyses were performed in four series over a 1-month period, each determined in quadruplicate to calculate the total coefficient of variation. The accuracy of the assay and its calibration was assessed using calibrator samples provided by an international proficiency testing scheme for sirolimus (Dr. D.W. Holt, The Analytical Unit, St. George's Hospital Medical School, London, UK) over a 6-month period; the declared target concentrations and the results of the analyses were compared by linear regression.

During routine use the whole blood pool from residual samples of sirolimus-treated patients was used in each series as a precision control. Additionally, for quality control, the spiked whole blood samples in a low, medium, and high concentration range (5.1, 10.2, and 20.4 µg/l) were regularly measured; they were prepared from a stock solution that was separately weighted-in; inaccuracy below 10% for the control samples was defined acceptable for routine analysis.

To test the specificity of the method, 10 whole blood samples from transplant patients treated with cyclosporin, tacrolimus, or mycophenolat mofetil were analyzed without addition of the internal standard; generation of a signal in the MRM traces of sirolimus and the internal standard, respectively, was assessed. Moreover, 50 whole blood samples from non-transplant intensive care unit patients – each treated with several drugs – were analyzed in the same way.

Results

The mean recovery of the extraction procedure was 73% for sirolimus and 75% for desmethoxy-rapamycin (internal standard) at a concentration of 7.5 µg/l, 77% for sirolimus and 74% for desmethoxy-rapamycin at a concentration of 15 µg/l, and 73% for sirolimus and 76% for desmethoxy-rapamycin at a concentration of 30 µg/l.

The assay proved to be linear over the full calibration range (0–50 µg/l) with $r > 0.999$ in four series of a pre-routine validation. Recalculated concentrations of the calibrator samples (mean) were as follows: calibrator 25 µg/l, 102.5%; calibrator 12.5 µg/l, 98.3%; calibrator 6.25 µg/l, 99.4%; calibrator 3.1 µg/l, 103.6%; calibrator 1.6 µg/l, 102.3%; the signal-to-noise ratio in the latter sample was approximately 16:1.

Close agreement was found in 12 analyzed calibrator samples obtained from an international proficiency testing scheme between found and target sirolimus concentrations yielding the following results of linear regression analysis: sirolimus found = $1.02 \times$ sirolimus target - 0.3 µg/l (concentration range 0 to 60 µg/l).

The total coefficient of variation ($n=16$) was 8.2% for the low pool (1.8 µg/l), 5.3% for the medium pool (13.1 µg/l), and 4.9% for the high pool (21.2 µg/l), with the pre-

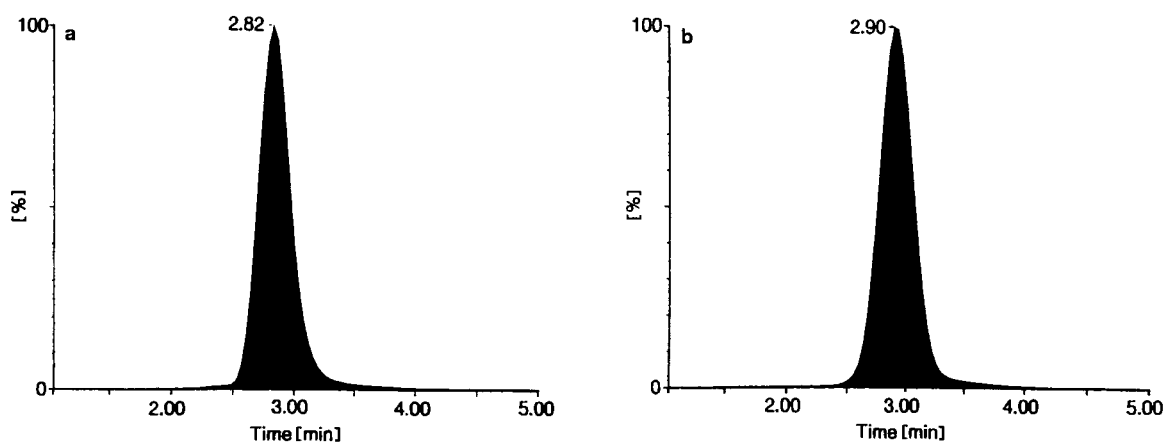


Figure 2 Representative multiple reaction monitoring chromatogram of a whole blood sample from a heart transplant patient (sirolimus concentration 7.8 $\mu\text{g/l}$). (a) m/z 931.5>864.5

represents the transition of sirolimus, (b) m/z 901.5>834.5 represents the transition the internal standard desmethoxy-rapamycin.

liminary therapeutic range 5 to 15 $\mu\text{g/l}$. For the whole blood pool used for quality control in routine application with sirolimus concentration of 13.1 $\mu\text{g/l}$, 1-month coefficients of variation were constantly below 7% and control samples remained within the 10% inaccuracy range.

In none of the samples from patients not treated with sirolimus a specific peak signal was observed. Pure multiple reaction chromatograms for sirolimus and the internal standard were obtained from samples from sirolimus-treated patients (Figure 2); no additional peaks were found and the peak shape was identical to that of calibrators with an equally low background signal.

After 6 months of routine use and approximately 3000 clinical samples not a single instrument malfunction occurred, and the only maintenance performed was a single cleaning of the ion source cone requiring about 30 min; venting of the system was not necessary for this maintenance thanks to a vacuum blocking valve in the Micromass ion source. The analytical column was washed in back-flush with methanol overnight once a week. The results of the analytical chromatography proved remarkably constant with respect to retention time and peak shape. After more than 3000 analyses the Waters Oasis HLB[®] extraction column did not show any signs of reduced extraction efficiency or increased back pressure.

Discussion

Here we describe a rapid LC-tandem MS method for the determination of sirolimus in whole blood that applied atmospheric pressure electrospray ionization after manual sample deproteinization step followed by on-line solid-phase extraction and liquid chromatographic separation of the extracts. In contrast to the previously described LC-tandem MS method (24) we used a separate extraction column packed with a copolymer material for on-line sample clean-up and

elution of the extracts from this extraction column in a back-flush mode onto the series-connected analytical column *via* column-switching. The method proved to be efficient, linear, and accurate as well as free of relevant ion suppression effects. In routine use the method was precise and rugged, and the instrumentation was found most reliable.

Compared to previously described methods, the combination of LC-tandem MS technology with on-line solid phase extraction enables quantification of sirolimus with a considerably shorter hands-on time (23) and running time (18). Thus, this method could be applied to large series of clinical samples.

Protein precipitation with a mixture of methanol and zinc sulfate was most efficient and resulted in completely clear supernatants; protein precipitation was combined in one step with the addition of the internal standard. For the subsequent on-line solid-phase extraction of the supernatants a copolymer material was used (Waters Oasis HLB[®]). This macroporous material is formed by equimolar polymerization of lipophilic *n*-vinylpyrrolidone monomers and hydrophilic divinylbenzyl monomers and thus possesses hydrophilic as well as lipophilic retention characteristics. The Oasis HLB[®] extraction column (HLB for hydrophilic-lipophilic-balance) can be operated at high flow rates (up to 4 ml/min) leading to an efficient sample preparation within a short time. The extraction columns proved remarkably stable with no apparent loss of extraction efficiency or increase in back-pressure after more than 3000 analyses. The highly efficient sample clean-up resulted in stable chromatography results with minimal background signal, constant retention times and peak shape. Moreover, visual fouling on the electrospray source components was minimal and cleaning (which did not require the venting of the system) was necessary only after extended working periods.

The Z-spray[®] system of the Micromass instrument yields stable mass-spectrometric results; this system forces the ion beam into two orthogonal directions by application of a counter-charge; in contrast,

non-ionized compounds of the electrospray are not deviated and are thus effectively filtered out before entering the high-vacuum area of the mass spectrometer.

Despite the efficient two-step sample clean-up (precipitation and on-line extraction), chromatography should not be omitted in LC-tandem MS analyses for the sake of even shorter analytical running times since ion suppression may arise from residual matrix components (25, 26).

The method described here can be easily modified to quantify cyclosporin and tacrolimus simultaneously by simply adding the respective multiple reaction monitoring traces; pre-routine validation revealed analytical performance for these analyses similar to the determination of sirolimus; for logistical reasons, however, the determination of these two immunosuppressants is still performed by immunoassay in our laboratory.

Volosov *et al.* (24) have recently reported a LC-tandem MS method for the quantification of sirolimus which – similarly to our method – employs on-line solid-phase extraction. In this method, however, no separate extraction column was used. The analyte was retained on a single HPLC column, matrix components were washed with ammonium acetate buffer and after post-column switching the analytes were eluted to the mass spectrometry system (Sciex API 2000) by changing to the methanolic mobile phase. Direct comparison of this method with our method described above would be difficult. However, the principle applied in our method (a separate extraction column requiring only one additional HPLC pump) has the advantage that practically matrix-free extracts are loaded onto the analytical column; this explains our very constant chromatographic results. Also, the ion-suppression effects from residual matrix components are further minimized contributing to our favorable experience with the method.

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