Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Sirolimus quantification by high-performance liquid chromatography with ultraviolet detection

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Keywords
high-performance liquid chromatography, sirolimus, ultraviolet detection, whole blood.

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Received: 4 May 2004
Revised: 8 July 2004
Accepted: 4 October 2004

doi:10.1111/j.1432-2277.2004.00066.x

Summary

The need to adapt optimal conditions of sirolimus blood level monitoring in laboratories led us to optimize an high-performance liquid chromatography–ultraviolet method and compare the elution performances using the mobile phase A, 68% MeOH/2% acetonitrile (ACN)/30% H_2O and mobile phase B, 30% MeOH/42% ACN/28% H_2O. Samples were assayed with 1-chlorobutane, redissolved in MeOH/water and injected onto a C-18 column at 50 °C. The assay achieved sensitivity of 2.5–150 ng/ml (CV = 10.6%) and recovery of 92–103.6%. The intra- and interassay precisions ranged from 3.3% to 13% and from 5.9% to 15% for quality controls of 7.5, 60 and 120 ng/ml. The mobile phase A was unable to elute and recover sirolimus and internal standard in the expected retention time and concentration. Under our working conditions, the assay was precise, accurate and sensible, stressing the importance of establishing for the best working conditions according to the staff and demands of the laboratory.

Introduction

Sirolimus (rapamycin) is an immunosuppressive agent used in combination with cyclosporine and corticosteroids to prevent acute rejection in renal allograft transplant recipients [1–3].

Sirolimus is a critical dose drug, showing significant inter- and intra-individual pharmacokinetic variability and drug interactions, precluding the use of fixed dose regimens. Through whole blood concentrations correlate with exposure [area under the curve (AUC)] and with efficacy and/or toxicity. Therefore, the use of therapeutic drug monitoring (TDM) is required to adjust doses to reach target therapeutic concentrations and to follow patients' compliance [4].

Chemically, this antibiotic isolated from *Streptomyces hygroscopicus* is a cyclic 31-membered macrolide, and has an ultraviolet (UV) absorption maximum at 278 nm [1–3]. Because of its high partition into red blood cells, sirolimus has been measured in whole blood instead of plasma [5] being ethylenediamine-tetraacetic acid (EDTA) the preferred anti-coagulant to minimize clotting problems [3].

Different methods have been developed to determine sirolimus blood concentrations, including an automated immunoassay and high-performance liquid chromatography (HPLC) with UV or mass spectrometric (MS) detections [1,2,6,7].

The immunoassay, although automated, usually super estimates sirolimus blood concentrations. The antibody used in this immunoassay shows cross-reactivity with some sirolimus metabolites, resulting from demethylation, hydroxylation, or both, and with sirolimus isomers [8].

The HPLC–UV methods show good sensitivity for sirolimus whole blood therapeutic concentration ranges and are more available in clinical and reference laboratories than in HPLC–MS [1]. Samples extracted from biologic matrices may show chromatographic interferences, one of the most routine problems associated with HPLC–UV assay, requiring clean-up extraction procedures to reduce and/or remove proteinaceous and other substances that absorb UV light at 280 nm. Different mobile phases may increase recovery and also eliminate some late eluting substances.

This study was performed to: (i) optimize an HPLC method with UV detection for sirolimus quantification in whole blood and (ii) compare the performances of sirolimus and internal standard elutions using two different mobile phases: mobile phase A consisting of 68% methanol (MeOH), 2% acetonitrile (ACN) and 30% H_2O ('reference method' [2]), and mobile phase B consisting of 30% MeOH, 42% ACN and 28% H_2O.

Materials and methods

Reagents

ACN, 1-chlorobutane, MeOH, all of HPLC grade, and sodium acetate, analytical grade were from Merck (Darmstadt, Germany). Drug-free whole blood collected in EDTA was obtained from local blood bank for preparation of assay calibration standards, quality controls (QC) and blanks.

Desmethoxysirolimus [internal standard (IS)] and sirolimus were obtained from Wyeth-Ayerst Research Laboratories (Princeton, NJ, USA). An IS stock solution (100 μg/ml) was prepared in MeOH and diluted in water to obtain a working solution of 2.5 μg/ml. To prepare calibration standards, sirolimus was weighed and dissolved in MeOH to obtain a stock standard solution of 250 μg/ml. To produce a consistent matrix for the whole-blood hemolysate, two substock solutions (0.25 and 2.5 μg/ml) were prepared in water. Calibration standards containing 2.5, 5, 10, and 25 ng/ml or 50, 75, 100, and 150 ng/ml were prepared using the 0.25 or the 2.5 μg/ml substock solution, respectively. A blank (containing no added sirolimus) was also prepared and run with each batch of samples. A different stock solution (300.0 μg/ml) was used to prepare quality controls. A substock (3.0 μg/ml) was prepared by diluting the stock solution in water. Three concentrations of sirolimus QC in whole-blood hemolysates were then prepared from the substock. QC samples were assayed in parallel with clinical samples to monitor assay performance. Nominal concentrations for QC material were 7.5, 60.0 and 120.0 ng/ml. All whole blood calibration standards, QC and blank samples and stock solutions in methanol were stored at −70 °C.

Extraction procedure

Aliquots of 1 ml standards, blank (drug-free whole blood) and quality controls were thawed at room temperature and labeled in 15 ml round bottom borosilicate glass tubes with teflon-coated screw caps containing 3 ml of 0.1 M sodium acetate buffer (pH 4.7), 50 μl 2.5 μg/ml IS, and 7 ml of 1-chlorobutane. The tubes were shaken for 1 h on a reciprocal shaker at 250 shakes per minute and centrifuged (Allegra 6R; Beckman Coulter, Fullerton, CA, USA) at 3000 rpm (2500 *g*) for 5 min at 4 °C. All tubes were placed upright in a dry ice/methanol bath for 5 min or until the pellet became frozen. The solvent layer was pipetted into a clean borosilicate glass conical tubes and evaporated to dryness at 45 °C under nitrogen gas or in a Speed Vac SC 110 (Savant Instruments, Holbrook, NY, USA). Aliquots of 300 μl of a mixture of MeOH/water (70:30) were added to the tubes and vortexed for 1 min at high speed and sonicated (Elma Ultrasound Bath; Lab-Line Instruments, Inc, Melrose, IL, USA) for 5 min. The tubes were centrifuged (10 min, 2500 *g*, 4 °C) and the extract (100 μl) was injected directly onto the analytical column in an HPLC system.

HPLC conditions

The HPLC was performed using Shimadzu equipment (Shimadzu Corporation, Kyoto, Japan) consisting of two LC-10AD *VP* pumps, one CTO-10AS *VP* column oven feted with a Rheodyne loop injector, and one SPD-10A *VP* UV detector. The conditions for the system were as follows: precolumn, Supelguard LC-18 (Supelco column, 2 cm cartridge) (Sigma-Aldrich, Bellefonte, PA, USA); separation column, Supelcosil LC 18 (15 cm × 4.6, 3 μM) (Supelco column) heated at 50 °C; the mobile phase, 30% MeOH/42% ACN/28% H_2O, eluted isocratically at 1 ml/min. UV detection was made at 278 nm, with sensitivity setting of 0.010 AUFS (absorbance units full scale).

A gradient wash of 100% ACN for 5 min was applied after each sample injection to elute possible late eluting peaks from the analytic column. Next, the column was re-equilibrated with the mobile phase described above for 10 min.

Retention time was used to identify peaks of interest. The Class-VP 5.03 software (Shimadzu Corporation) was used to analyze the data, using calculations based on peak area, defined as the sirolimus to internal standard peak area ratio. The program performs a multilevel linear calibration based on the least squares analysis of the standards curve. Calibration samples were omitted from the calibration curves when a ±20% bias was observed.

Calibration curves were excluded whenever the correlation coefficient was inferior to $r^2 = 0.98$. The performance of the assay was assessed daily by using one or two sets of quality controls and a particular run was discarded when a 20% bias was observed in any QC level.

Mobile phase comparison

Elution was carried out using two different mobile phases: mobile phase A consisting of 68% MeOH, 2% ACN and 30% H_2O and mobile phase B consisting of 30% MeOH, 42% ACN and 28% H_2O. In all studies described below, the mobile phase B was employed.

Precision study

Intra-assay precision was determined by performing 10 replicates at each QC level in a single analytical run. Interassay precision was assessed by analyzing the QC samples five times on five successive days.

Sensitivity

Sensitivity was assessed by performing 11 replicates of the lowest standard (2.5 ng/ml) in a single run and comparing them with the nominal concentration.

Analytical recovery

Studies of recovery were performed using the analytical standard curve. Four samples of each concentration were analyzed in a single analytical run and compared with the nominal concentrations.

Results and discussion

The need to use sirolimus therapeutic monitoring in clinical transplantation has motivated the development of methods to measure its concentration in whole blood. As an automated immunoassay still awaits the identification of an antibody with low cross-reactivity with sirolimus metabolites, HPLC with UV detection has been the most utilized method in many transplant centers. Depending on the drug combination, time of post-transplant and time of blood collection, sirolimus therapeutic concentrations may range from 3 to 5 to 50–70 ng/ml.

In this study, we first reproduced the HPLC method described by Maleki *et al.* [2]. When the mobile phase A was used in the column temperature set at 60 °C, under our conditions and using our equipment, sirolimus and IS could not be eluted with the expected retention time or with expected peak area (Fig. 1a). Then, modifications were tested to optimize the method to our local

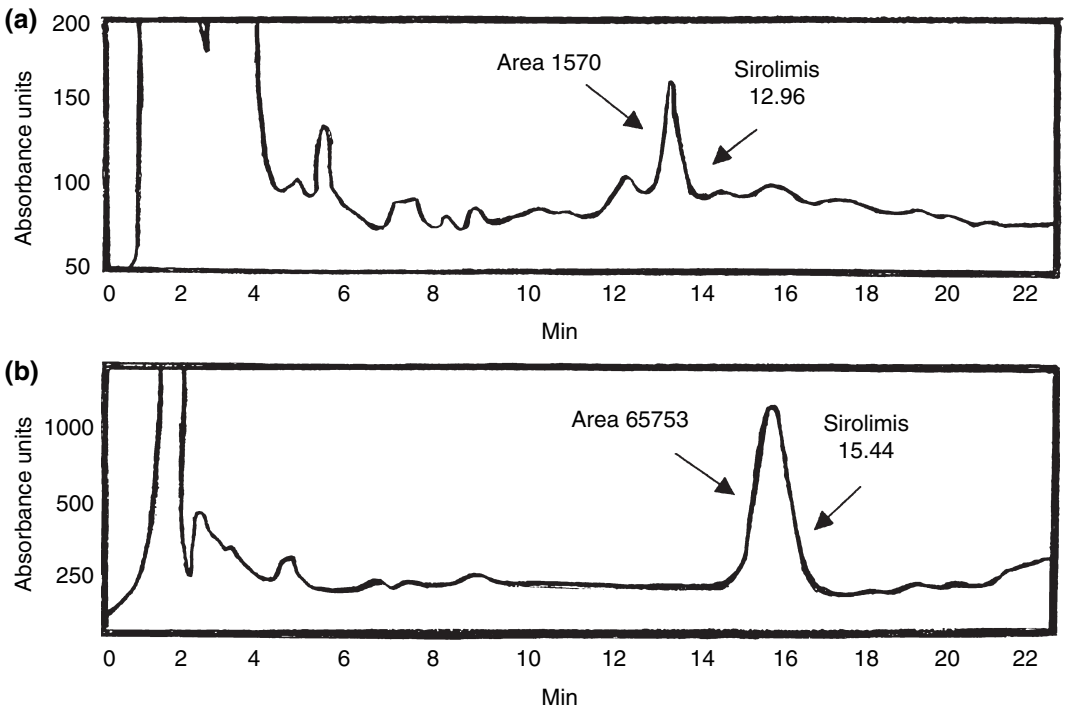

Figure 1 Representative chromatograms for sirolimus eluted with (a) 68% MeOH/2% ACN/30% H_2O; (b) 30% MeOH/42% ACN/30% H_2O. Arrows indicate retention time in minutes.

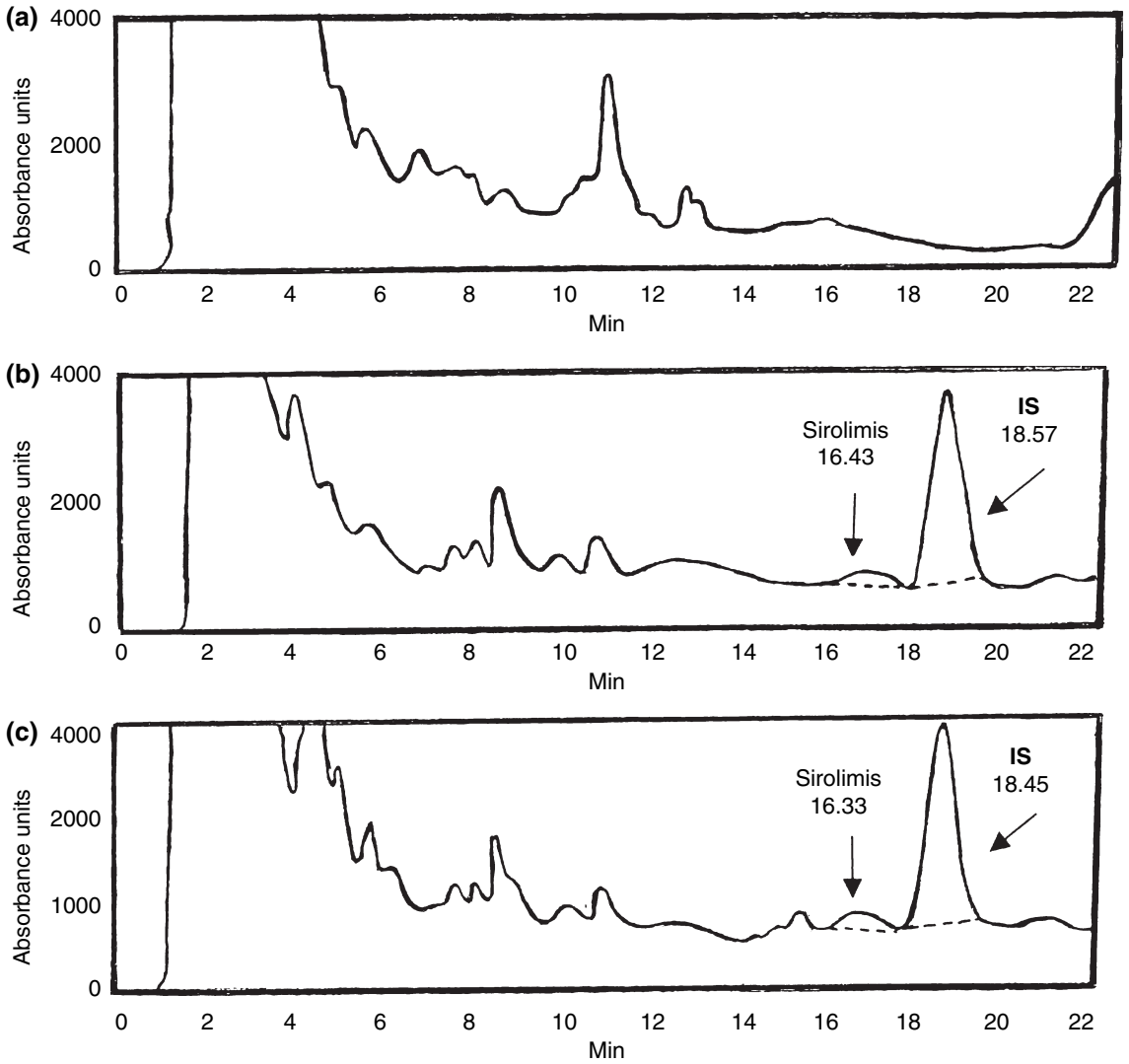

Figure 2 Representative chromatograms for (a) the extracted blank; (b) the 2.5 ng/ml calibration level; (c) whole blood extracted patient sample containing 5.2 ng/ml of sirolimus. *IS, internal standard. Arrows indicate retention time in minutes.

conditions. First, the mobile phase mixture was changed to 30% MeOH, 42% ACN and 28% H_2O and the column temperature was maintained. As no improvement was observed, we started a series of tests, including different column temperatures and changes in reconstitution solution and mobile phase (Table 1). Finally, to improve the chromatographic profile two modifications were made: (i) the column temperature was changed to 50 °C and (ii) mobile phase A (68% MeOH, 2% ACN and 30% H_2O) was changed to mobile phase B (30% MeOH, 42% ACN and 28% H_2O).

With the mobile phase B, it was possible to elute and recover sirolimus and IS as shown in Fig. 1b. In addition, the retention times for both peaks provided a good separation for the quantification of sirolimus. Figure 2 shows representative chromatograms for the blank A, lowest detectable standard (2.5 ng/ml) B, and patient sample contained sirolimus (c).

Table 1. Precisions of the present method for the determination of sirolimus in whole blood.

	Intra-assay CV (%)	Interassay CV (%)
7.5 ng/ml	13.0	15.0
60.0 ng/ml	7.5	9.6
120.0 ng/ml	3.3	5.9

CV, coefficient of variation.

Table 2. Recovery of extracted sample.

	Target (ng/ml)							
	150	100	75	50	25	10	5	2.5
Mean ± SD	155 ± 8	102 ± 8	77 ± 1	51 ± 4	24 ± 2	9 ± 0.6	5 ± 0.5	2.6 ± 0.3
CV (%)	5.3	7.4	1.5	7.4	6.4	7.0	9.5	11.3
Recovery (%)	103.6	101.8	102.1	101.3	94.2	92.0	100.6	102.4
Bias (%)	3.6	1.8	2.1	1.3	−5.8	−8.0	0.6	2.4

Table 3. Method comparison and modifications tested to improve chromatographic profiles.

	Current method	Reference method
Sample volume (ml)	1.0	1.0
Reconstitution solution	70% MeOH/30% H_2O	70%MeOH/30% H_2O
Mobile phase	30% MeOH/42% ACN/28% H_2O B	68% MeOH/2% ACN/30% H_2O A
Column temperature	50 °C	60 °C

It is often advantageous to use ternary mixtures containing water and two organic solvents, and their compositions are chosen to optimize and to improve selectivity. Organic modifiers not only affect the gross properties of the mobile phase (through changes in surface tension, dielectric constant, viscosity, etc.) but also interact in specific way with the compounds, stabilizing or deforming their structures. Perhaps the chosen mobile phase (30% MeOH, 42% ACN and 28% H_2O), with a more equilibrated mixture of ACN and MeOH, induced structural modification in the compounds that allowed an increase in surface contact with the stationary phase (column properties). Remembering that, besides the MeOH (in high concentrations) capacity of inducing either reversible or nonreversible structural deformation, ACN is more efficient in decreasing the relative retention of a particular compound than MeOH, generally causing higher mass recoveries. In addition, we must take into account the fact that the HPLC equipment itself could be an additional source of variance.

Calibration was in the range of 2.5–150 ng/ml. The lower limit of quantification was set at 2.5 ng/ml, the value of the lowest calibrator. Mean value was 2.9 ± 0.3 with a CV of 10.6% and a bias of 16.8%.

Sirolimus recovery from whole blood samples, determined by comparison of mean observed and nominal target values at each level, varied from 92% to 103.6% (Table 2).

The concentrations at which the precision study was assessed were 7.5, 60 and 120 ng/ml. The intra-assay (repeatability) presented mean values of 8.1 ± 1.1, bias 7.7% (7.5 ng/ml), 61.2 ± 4.6, bias 2.0% (60 ng/ml) and 122.3 ± 4.1, bias 1.9% (120 ng/ml), respectively. Similarly, the interassay (reproducibility) presented 7.4 ± 1.1, bias −1.7% (7.5 ng/ml), 59.9 ± 5.8, bias −0.2% (60 ng/ml) and 121.7 ± 7.2, bias 1.5% (120 ng/ml), respectively (Table 3).

To summarize, using our temperature conditions (50 °C) and elution (30% MeOH/42% ACN/28% H_2O), the complete extraction procedure takes approximately 4 h and the HPLC–UV assay achieved high analytical sensitivity (2.5 ng/ml) with acceptable imprecision (<15%) and demonstrated good recovery within the range of sirolimus concentrations (2.5–150 ng/ml) used in clinical practice.

Although many studies have reported on sirolimus whole blood quantification by HPLC or other strategies, this present study allows us to stress that each laboratory needs to develop its own optimal working conditions, according to its staff and demands.

Acknowledgements

Wyeth-Ayerst Research Laboratories, São Paulo, Brazil, funded this work. The authors thank Luciana Cristina Teixeira for general assistance.

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