Determination of sirolimus in blood by high-performance liquid chromatography with ultraviolet detection - Experience and review

Determination of Sirolimus in Blood by High-Performance Liquid Chromatography with Ultraviolet Detection – Experience and Review

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ABSTRACT

Sirolimus (SRL) is a potent immunosuppressant. Therapeutic drug monitoring (TDM) of SRL is required to optimize the therapy. Immunoassay, high-performance liquid chromatography with ultraviolet detection (HPLC-UV), and HPLC with mass-spectrometric detection (HPLC-MS or HPLC/MS/MS) have been used in the analysis of SRL. The purpose of this study was to share our experience in validating a HPLC-UV method for analyzing SRL and to have an overview of HPLC-UV methods used in SRL assay. A validated HPLC/UV method developed by Wyeth-Ayerst Research with minor modification was use to determine SRL concentration in human whole blood. An Alltima C18 column (5 µm, 150 × 2.1 mm) was used as the stationary phase. The mobile phase was 60% acetonitrile in water, and the flow rate was 0.5 mL/min. Samples were prepared by spiking human whole blood (0.5 mL) with the internal standard (IS) and designated amount of SRL, except blank. Zinc sulfate (50 g/L, 1 mL) and acetone (1 mL) were used for hemolysis and deproteinization. After alkalizing supernatant with 100 mM NaOH (0.2 or 0.3 mL), 1-chlorobutane (2 mL) was used for extraction. The 1-chlorobutane layer was dried, reconstituted with mobile phase and back extracted with 0.5 mL of *n*-hexane. The limitation of quantification was 2.5 ng/mL and the standard curve was linear at the concentration range of 2.5-75 ng/mL. The intraday and interday coefficients of variation were 2.1-5.2% and 2.8-5.7%, respectively. The intraday and interday relative errors were -0.03-5.3% and 0.7-3.3%, respectively. The recoveries for SRL and the IS were 78.5-92.8% and 76.9 ± 3.9 %, respectively. The samples were proven to be stable after 3 freeze/thaw cycles, and the extract was stable over 24 hr at an autosampler set to 4°C. In addition to an overview of the chemical properties of SRL, different HPLC-UV methods for the quantification of SRL were provided to identify analytic parameters that are critical for the establishment of HPLC-UV assay for SRL.

Key words: immunosuppressive, high-performance liquid chromatography, sirolimus, therapeutic drug monitoring, transplantation

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INTRODUCTION

Sirolimus (Rapamycin, AY-22989, Wy-090217, SRL), a lactone-lactam macrolide antibiotic derived from Streptomyces hygroscopicus[1], is a potent immunosuppressive agent that was approved by the U.S. Food and Drug Administration (FDA) for use with cyclosporine- or tacrolimus-based immunosuppression in kidney transplantation[2,3]. In addition, the European regulatory authorities have approved the drug for use in combination with corticosteroids in kidney transplantation[3]. SRL has not only a considerable inter- and intra-individual variability (about 8 folds) in clearance, but also sub-optimal correlations between blood concentrations and dose or demographic features[4]. Fortunately, the excellent correlation between steady-state trough concentration (C_0) and area under the concentration-time curve (AUC) makes C_0 a simple and reliable index of SRL exposure[5]. The target ranges of SRL C_0 start at 5 ng/mL[3]. A clear relationship exists

between C_0 of SRL and efficacy and toxicity[5]. Therapeutic drug monitoring (TDM) of SRL is important to optimize its therapeutic effects[6,7]. Several types of analytical methods have been used in TDM of SRL, including immunoassay, high-performance liquid chromatography with ultraviolet detection (HPLC-UV), and HPLC with mass-spectrometric detection (HPLC-MS or HPLC/MS/MS)[8]. For the phase III clinical trials, an investigational microparticulate enzyme-linked immunoassay (MEIA, Abbott Laboratories, Abbott Park, Illinois) was used[9,10]. The quantification limits were 3.0–22 ng/mL, not interfered by cyclosporine (CsA), tacrolimus (FK) or mycophenolate mofetil[9,10]. A positive mean bias of 41.9% when compared with HPLC-MS is potentially attributable to the interference of SRL metabolites[9]. However, for reasons unrelated to its technical performance, the kit was not commercially available[3]. HPLC-UV and HPLC/MS/MS spectroscopy are the methods of choice for determination of whole-blood SRL[11]. The former method has a sensitivity and lower limits of quantification (LOQ) of < 1 ng/mL, while the latter have sensitivities of 0.4-6.5 ng/mL and LOQ of 1-6.5 ng/mL[11-18]. Although HPLC-UV methods

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use a less sophisticated mode of detection than HPLC-MS or HPLC/MS/MS methods and are complicated by interfering peaks in the chromatograms, these two HPLC methods provide similar results[8]. In addition, HPLC-UV is more readily available in clinical and reference laboratories. As the absorption peak of SRL is relatively small, chromatographic interference from temperature, test tubes, biologic matrices and chemical solvent make assay of SRL by HPLC-UV a challenge to many laboratories. The goal of this study was to share our experience in validating an HPLC-UV method for analyzing SRL, as well as to give an overview of HPLC-UV methods used in SRL assay.

MATERIALS AND METHODS

A validated method developed by Wyeth-Ayerst Research (Princeton, NJ)[18] with minor modification was used in our lab. The validation was performed in accordance with the regulatory guidelines of the U.S. FDA[19].

I. *Reagents and Chemicals*

SRL was obtained form Wyeth-Ayerst Analytical R & D (Pearl River, NY). The internal standard (IS, 32-desmethoxy rapamycin) was obtained from Wyeth-Ayerst Research (Princeton, NJ). HPLC grade solvents, such as methanol (MeOH), acetone, 1-chlorobutane (C_4H_9Cl) and acetonitrile (CH_3CN), were purchased from Merck KgaA (Darmatadt, Germany). *n*-hexane (HPLC grade) and sodium hydroxide (NaOH) 50% solution were purchased from Mallinckrodt Baker. Inc. (Phillipsburg, NJ, USA). Pure water was prepared by Millipore Direct-Q^{TM}. Zinc sulfate heptahydrate (GR reagent, 99.5% minimum purity) was purchased from RdH Laborchemikalien GmbH & Co. KG. (Seelze, Germany).

II. *Equipment*

Vortex mixer (Thermolyne, MAXI-MIX II, Dubuque, Iowa, USA) and large capacity vortexer (Glas-Col, 099A-LC1012, Indiana, USA), sonicator (Elma-Hans Schmidbauer GmbH & Co KG, T710DH, Singen, Germany), centrifuge (Kubota Corporation, KN-70, Osaka, Japan) and nitrogen gas blower/dry thermo bath (Eyela, MGS-1000, Tokyo, Japan) were used during the extraction procedure. A HPLC system (Agilent Technologies Inc., 1100 LC, Palo Alto, California) consisted of a pump (G1311A), degasser (G1322A), photodiode array (PDA)-detector (G1315B), autosampler (G1329A), autosampler temperature controller (G1330A) and thermostat column compartment (G1316A) was used to analyze the samples.

III. *Preparation of Calibrator and Quality Control (QC) Samples*

All the stock or spiking solutions were prepared in glass volumetric flasks, then transferred to glass or polypropylene (PP) tubes, and stored at -80°C when not in use. Stock standard of SRL (100 µg/mL) was prepared in MeOH. The IS spiking solution was prepared at a concentration of 250 ng/mL in 50% methanol water.

Calibrators and quality-control (QC) samples were prepared in potassium EDTA human whole blood (Biological Specialty Corporation, Colmar, PA, USA) using stock standard solutions. Calibration curves contained seven non-zero calibrators assayed in duplicate (nominal SRL concentrations: 2.5, 5, 10, 15, 25, 50 and 75 ng/mL). The correlation coefficient (r) between concentration and the peak height ratio should be ≥ 0.98. Recalculated calibration data should be within ± 20% of the nominal value. A maximum of two calibration standards (at the different concentrations) might be removed as outliers.

IV. *Extraction Procedure*

The whole blood sample (0.5 mL) was transferred to a 16 × 100 mm screw-cap (Teflon lined cap, Corning Inc., Corning, NY, USA) glass culture tubes, spiked with 75 µL of IS, and vortex-mixed for 1 min. One milliliter of Zinc sulfate (50 g/L) and 1 mL of acetone were added to each tube. The tubes were capped, vortex-mixed for 1 min, and centrifuged at 3000 rpm and ambient temperature for 7 min. The supernatant was poured off into a 16 × 100 screw cap tube. NaOH (200 µL or 300 µL, 100 mM) was added to each tube and vortex-mixed briefly. 1-chlorobutane (2 mL) was added to each tube. The tubes were capped, vortex-mixed for 1min, and centrifuged at 3000 rpm and ambient temperature for 7 min. The upper organic layer was transferred to a 10 mL conical screw cap centrifuge tube (Kimble Glass Inc., Vineland, NJ, USA), and then dried under nitrogen at ambient temperature for approximately 30 min. The dried extracts were reconstituted with 150 µL of mobile phase, capped and vortex-mixed for approximately 20 sec. *n*-hexane (500 µL) was added to each tube for back-extraction. The tubes were capped with Teflon-lined caps, vortex-mixed for approximately 30 sec, and centrifuged for 3 min at 3000 rpm and ambient temperature. After removing and discarding the upper hexane layer from each sample, the extracts were dried under nitrogen at ambient temperature for 1 min to remove last traces of hexane. The extracts were transferred to 250 µL conical glass inserts in 1.5 mL autosampler amber glass sample vials. The vials were capped with screw-polypropylene cap with Teflon/silicone rubber septa (Sun International, Wilmington, NC, USA) and placed on the HPLC autosampler.

V. *HPLC Analysis*

The analytical column was Alltima C18 column (150 × 2.1 mm, particle size 5 µm from Alltech Associates, Inc. USA) with Alltima C18 column guard column (7.5 × 2.1 mm, particle size 5 µm from Alltech Associates, Inc.

USA). The autosampler had temperature controller (set to 4°C) and equipped with column oven set at 50°C. The mobile phase was a mixture of CH_3CN 60% in H_2O, and injection volume was 50 µL. The flow rate was 0.5 mL/min which led to a typical pressure of 90-120 bars (1305-1740 psi). SRL and the IS were detected by UV absorption at 278 nm at retention time of approximately 9 and 11 min.

Measured (or calculated) SRL concentrations (ng/mL) were obtained from the weighted linear regression line relating to the peak height ratios of SRL/IS (Y) to the concentration ratios of SRL/IS (X), with weight = 1/(SRL concentration).

VI. *Accuracy and Precision*

Accuracy and precision were assessed using low (7.5 ng/mL), medium (22.5 ng/mL), and high (58.0 ng/mL) QC levels. Each level was assayed in six replicates in a single day for intraday determination and as six replicates over three separate days to determine the interday inaccuracy (relative error) and imprecision (coefficient of variation, CV). Relative error and imprecision should be within ± 15% for the two highest concentrations and ± 20% for the lowest concentration.

VII. *Upper and Lower Limits of Quantification (ULOQ and LLOQ)*

ULOQ and LLOQ were estimated by analysis of six replicates at the highest (75.0 ng/mL) and the two lowest calibration standards (2.5 ng/mL and 5.0 ng/mL).

VIII. *Recovery*

The recovery of SRL was determined at three QC levels. The recovery of the IS was determined at a nominal concentration of 32.6 ng/mL (0.5 mL whole blood spiked with 75 µL of 250 ng/mL IS). Peak height measurements obtained from the extracted samples were compared with the peak height measurements obtained from unextracted samples prepared in mobile phase. Mean and standard deviation were calculated from at least five measurements at each level.

IX. *Sample Stability*

The stability of SRL in human EDTA whole blood spiked after freeze-thaw cycles was determined at the nominal concentration of 15 ng/mL. The frozen temperature was -80°C.

X. *Stability of Sample Extracts*

Stability of the sample extracts was tested at three QC concentrations. These samples were extracted to yield a total volume of extract sufficient to allow aliquots for two injections. Six measurements were made at each concentration immediately after extraction and after storage in autosampler (set to 4°C) for 24 hr.

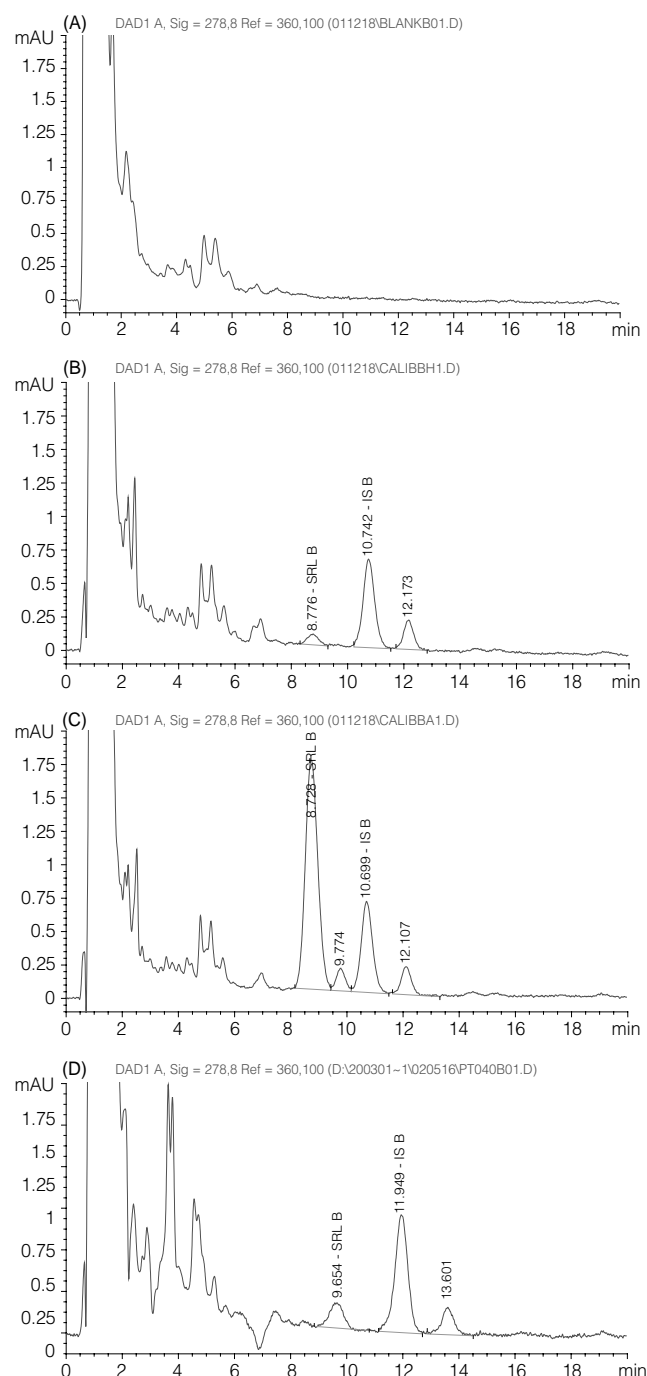**Figure 1.** Typical chromatograms of (A) drug-free whole blood, (B) extract of blank whole blood spiked with 2.5 ng/mL SRL and 32.6 ng/mL IS, (C) extract of blank whole blood spiked with 75 ng/mL SRL and 32.6 ng/mL IS and (D) extract of blood from a patient receiving SRL (4.45 ng/mL).
Both SRL and IS have two peaks (isomer B and isomer C). Peak of SRL isomer C can be clearly identified only at high concentration as in (C).

Table 1. Precision and accuracy in determining of SRL concentrations in human whole blood and limits of quantification (N = 6)

Sample (ng/mL)	Intraday			Interday		
	Mean ± SD	CV (%)	Accuracy (%)	Mean ± SD	CV (%)	Accuracy (%)
LLOQ (2.5)	2.2 ± 0.1	6.0	88.7			
Calibrator (5)	4.7 ± 0.4	9.4	94.3			
Low QC (7.5)	7.9 ± 0.4	4.6	105.3	7.7 ± 0.4	4.6	103.3
Mid QC (22.5)	22.5 ± 1.2	5.2	100.0	22.7 ± 1.3	5.7	100.7
High QC (58)	58.0 ± 1.2	2.1	100.0	58.9 ± 1.6	2.8	101.5
ULOQ (75)	68.6 ± 2.1	3.0	91.5			

LLOQ: lower limit of quantification; QC: quality control; ULOQ: upper limit of quantification.

Table 2. Recovery and stability of sample extracts (N = 6)

Nominal concentration	Recovery (%)		Stability of sample extracts (relative error, %)	
(ng/mL)	SRL[a]	IS[b]	Immediately after extraction	24 hr after extraction
7.5	89.1 ± 6.3		-0.8	3.0
22.5	84.8 ± 6.4		0.4	3.6
58.0	82.5 ± 3.2		0.9	4.7
32.6		76.9 ± 3.9		

[a]SRL: sirolimus.　　[b]IS: internal standard.

Table 3. Sample stability after freeze/thaw cycles (N = 6)

	Nominal concentration	Measured concentration		
	(ng/mL)	Mean ± SD (ng/mL)	CV (%)	Relative error (%)
Immediately after preparation	15.0	15.21 ± 0.81	5.3	1.4
1st freeze/thaw cycle	15.0	15.03 ± 0.28	1.8	0.2
2nd freeze/thaw cycle	15.0	13.39 ± 0.69	5.1	-10.7
3rd freeze/thaw cycle	15.0	15.62 ± 1.85	11.8	4.1

RESULTS

I. *Specificity*

The specificity of the method was demonstrated by the absence of interfering chromatographic peaks with retention times equal to those of SRL or IS in the extracts of blank human whole-blood samples. Representative chromatograms for extracts from a blank human whole blood sample, the lowest (2.5 ng/mL) and highest (75 ng/mL) calibrators, and a sample from a patient receiving SRL (4.45 ng/mL) are presented in Figure 1 (A), (B), (C) and (D), respectively. The retention time occasionally varied by 1 min or so after changing the column or solvent, but remained stable during the same run. Both SRL and IS have two peaks (isomer B and isomer C). Because isomer B is the main isomer, we use only the peak of isomer B for calculation. Isomer C, on the other hand, is a minority in various SRL formulations and its peak can be clearly identified only at high concentration.

II. *Accuracy, Precision and Limit of Quantification*

The CV and relative error values for the LLOQ were 6.0% and 11.3%, respectively, while those for ULOQ were 3.0% and 8.5%, respectively (Table 1). The precision is 3.0-9.4%. The correlation between concentration ratio and the peak height ratio were > 0.998. Intraday imprecision

(CV) and relative errors were 2.1-5.2% and -0.03-5.3%, respectively. Interday imprecision and relative errors were 2.8-5.7% and 0.7-3.3%, respectively (Table 1).

III. *Recovery and Stability of Sample Extracts*

The recovery of SRL from human whole blood ranged from 78.5% to 92.8%, and 76.9 ± 3.8% for the IS (Table 2), respectively.

The measured values immediately after extraction and more than 24 hr after extraction were within ±7.2% and ±10.9% of the expected value, respectively. The relative errors of mean measured values of the low, medium and high QC level immediately after extraction were -0.8%, 0.4%, 0.9%, respectively. Reanalysis of QC sample extracts after placing in autosampler for more than 24 hr showed corresponding relative errors of 3.0%, 3.6%, 4.7%, respectively (Table 2).

IV. *Sample Stability*

The measured values immediately after preparation and after freeze/thaw cycle (s) were all within ± 15.0% of the expected value. The relative error of mean measured value immediately after preparation was 1.4%. Reanalysis after the first, second and third freeze/thaw cycle showed corresponding relative errors of 0.2%, -11.6% and 4.1%, respectively (Table 3). All the CVs were less than 12%.

Table 4. Comparison of HPLC-UV methods used in SRL assay[12-18]

References	Napoli et al. (1994, 1996)	Yatscoff et al. (1992)	Maleki et al. (2000)	French et al. (2001)	Holt et al. (2000)	Svensson et al. (1997)
Lower detection limit (LOD)	1 ng/mL	1 ng/mL if 2 mL of blood used 0.5 ng/mL if 1 mL of blood used	2.5 ng/mL	2.5 ng/mL if 0.5 mL of blood used 1.25 ng/mL if 1 mL of blood used	6.5 ng/mL	0.4 ng/mL
Assay range	1-50 ng/mL	1-250 ng/mL	2.5-150 ng/mL	2.5-75 ng/mL	6.5-250 ng/mL	1-50 ng/mL
Internal standard (IS)	β-Estradiol-3-methyl ether	Demethyl-RAPA (DMRAP)	32-Desmethoxy RAPA	32-Desmethoxy RAPA	32-Desmethoxy RAPA	32-Desmethoxy RAPA
Stock standards of SRL	0.5 mg/mL in MeOH	4 µg/mL in 95% EtOH/H_2O	250 µg/mL in MeOH, dilute with H_2O to 2 substock: 25 µg/mL, 0.25 µg/mL	1 mg/mL in MeOH, dilute with 50% MeOH/H_2O to substock: 100 µg/mL		200 µg/mL in MeOH
Stock standards of IS	0.1 mg/mL in MeOH	8 µg/mL in 95% EtOH/H_2O	100 µg/mL in MeOH, dilute with water to 2.5 µg/mL	1 mg/mL in MeOH, dilute with 50% MeOH/H_2O to intermediate stock: 100 µg/mL, 2.5 µg/mL		400 ng/mL in MeOH
Storage of stock standards	-40°C, dark	-40°C		-80°C		-40°C
Precipitation Vortex mixed Centrifuge	No	No	No	5% $ZnSO_4$ 1mL, acetone1 mL 20 sec 3000 rpm, 5min	No	No
Liquid/liquid extraction	2 extractions	Single extraction with back extraction	Single extraction	Single extraction with back extraction	Single extraction with back extraction	Single extraction
1. blood	0.25-1 mL	2 mL	1 mL	0.5 mL	1 mL	1 mL
2. IS	0.1 mg/mL 20 µL	200 ng/mL 50 µL	2.5 µg/mL 50 µL	250 ng/mL 75 µL	100 µL	400 ng/mL 50 µL
3. extraction solvent	0.01 M Na_2CO_3 0.25-1 mL (= blood volume) tBME 7 mL × 2 MeOH 25-100 µL	13.3 g/L K_2CO_3 3 mL, $(C_2H_5)_2O$ 5.5 mL	0.1M CH_3COONa buffer (pH 4.7) 3mL C_4H_9Cl 7 mL	0.1 M NaOH 0.2 mL, 1- C_4H_9Cl 2 mL	tBME/ C_4H_9Cl/MeOH, 45/45/10, 4 mL	0.1 M CH_3COONa buffer (pH 4.7) 1 mL; C_4H_9Cl/$(C_2H_5)_2O$, 50/50, 7mL
4. vortex time	20 sec	30 sec	High speed 1 min, sonicated × 5 min	1 min	100 rpm × 20 min	
5. shaking time	15 min	10 min	1 hr on a reciprocal shaker at 250 shakes/min	No	No	Reciprocal shaking 30 min
6. centrifugation	1600 g × 5 min	3000 g × 5 min at autosampler vial	2000 g (3000 rpm) × 10 min, refrigerated centrifuge	3000 rpm × 5 min	1500 g × 10 min	5000 g × 10 min
7. dry ice bath			Upright, dry ice/methanol bath × 5 min to form a semi-frozen pellet			
8. layer used	tBME	$(C_2H_5)_2O$	C_4H_9Cl	C_4H_9Cl	tBME/C_4H_9Cl/MeOH	C_4H_9Cl/$(C_2H_5)_2O$
9. water bath	40°C		45°C	Ambient temperature	60°C	~40°C
10. evaporate to dryness	N_2 gas	N_2 gas	N_2 gas	N_2 gas	N_2 gas	N_2 gas
11. reconstitute solvent of dried extract	EtOH 150 µL × 2, dried then mobile phase 100 µL	Mobile phase 100 µL	70% MeOH/ H_2O, v/v	Mobile phase 150 µL	50% CH_3CN/H_2O, 0.5 mL, v/v	60% MeOH/ H_2O, 1 mL, v/v
12. back extraction	C_6H_{14} 0.1 mL	C_6H_{14} 0.1 mL		C_6H_{14} 0.5 mL	C_6H_{14} 0.5 mL	

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13. vortex time		30 sec		30 sec		
14. centrifuge		3000 rpm × 5 min		2600 g × 2 min	100 rpm × 10 min	100 rpm × 10 min
15. layer for analysis	Supernatant	Mobile phase (lower layer)	Supernatant	Mobile phase (lower layer)	1500 g × 10 min / Lower layer	–
Solid phase extraction	No	No	No	No	No	Yes
Chromatography						
Autosampler temperature	22°C		4 ± 2°C	4°C		
Sample for each analysis	85 µL	70 µL	130 µL	50 µL	200 µL	50 µL
Precolumn filter (guard column, column saver)	Upchurch 0.5 µm		C18 guard column & a column saver	C18 guard column		
Column	2 Suplecosil reverse-phase tandem C18 column (5 µm, 25 × 0.46 cm)	2 Spherisorb tandem C18 column (5 µm, 25 × 0.21 cm)	1 C18 analytical column (3 µm, 15 × 0.46 cm)	1 Alltima C18, analytical column (5 µm, 15 × 0.21 cm)	Ultrsphere C18-bonded silica column (5 µm, 25 × 0.46 cm)	Shandon Hypersil BDS C18 column (3 µm, 10 × 0.3 cm)
Column temperature	40°C	45°C	60 ± 2°C	50°C	50°C	70°C
Mobile phase	85% MeOH/ H_2O, v/v	70% MeOH/H_2O, v/v	MeOH/CH_3CN/H_2O (68/2/30)	H_3CN/H_2O (60/40)	65% CH_3CN/H_2O, v/v	MeOH/CH_3CN/ H_2O (34/38/28)
UV detection	276 nm	278 nm	278 nm	278 nm	278 nm	278 nm
Flow rate	1 mL/min	0.35 mL/min	1 mL/min	0.5 mL/min	1.5 mL/min	0.3 mL/min
Elution time	<40 min		35 min	20 min		
Elution time of SRL	~18-19 min		16 min	10.4 min	~22 min	~10 min
Flush solution	THF 1mL at 30 min via a 2-position actuator		100% CH_3CN			
Elution time of IS	~20-21 min		18 min	13 min	28 min	
Recovery of SRL						
Absolute recovery		35.3% ± 4.94%	88-106.3%	80.6%	81.5 ± 4.3%	~45%
Relative recovery	96%	~100%	80.0-88.8%	~100%		
Recovery of IS	70%			81.8%	62.7 ± 3.6%	~45%
Method of SRL concentration determination	SRL/IS peak-height ratio		SRL/IS peak-height ratio	SRL/IS peak-height ratio	Peak-area ratio of in-house control/analytes	
CV (within-day)	-21-27%	1.9-8.1%	12.0-14.4%	0.9-9.5%	3.1-5%	
CV (between-day)	6-17%	9.8-15.4%	2.6-13.0%	2.5-6.9%	0.4- 6.6%	9.8% at 5 ng/mL / 5.6% at 40 ng/mL
Relative error (within-day)	-9-14%		-0.2~ -4.8%	-3.7%-7.5%	-9.7%-9.2%	
Relative error (between-day)	-9-4%	9.8-14.4%	4.2-5.7%	1.1-5.2%		

Table 4. Comparison of HPLC-UV methods used in SRL assay[12-18]

References	Napoli et al. (1994, 1996)	Yatscoff et al. (1992)	Maleki et al. (2000)	French et al. (2001)	Holt et al. (2000)	Svensson et al. (1997)
Signal to a noise ratio	3.0 = 0.4 ng SRL	2.0				
Calibration curve	SRL 0, 2, 5, 10, 20, 30, 40, 50 ng/mL		SRL 0, 2.5, 5, 10, 25, 50, 75, 100, 150 ng/mL	SRL 0, 2.5, 5, 10, 15, 25, 50, 70, 75 ng/mL	SRL 0, 6.5, 13, 25.9, 51.8, 103.7, 207.4, 356.4 ng/mL	
Storage of sample extract	4°C overnight			-80°C	~22°C, 54 hr	

EtOH: ethanol.
(C$_2$H$_5$)$_2$O: diethyl ether.
CH$_3$CN: acetonitrile.
CH$_3$COONa: sodium acetate.
C$_4$H$_9$Cl: 1-chlorobutane (=butyl chloride).
C$_6$H$_{14}$: hexane.
H$_2$O: deionized water.
MeOH: methanol.
RPAP: sirolimus.
SRL: sirolimus.
tBME: t-butyl methyl ether.
ZnSO$_4$: zinc sulfate.

DISCUSSION

I. *Guidelines for TDM of SRL*

Definitive therapeutic levels of SRL remain to be established. Target C_0 depends on the concomitant immunosuppressive regimen and post-transplant interval. There are no conclusions on the most appropriate blood levels of calcineurin inhibitors (CNIs) and SRL when used in combination. A SRL level of 6-12 ng/mL is considered appropriated when combined with tacrolimus (FK)[20]. If cyclosporine (CsA) is being used at C_0 of 75-150 ng/mL, a SRL C_0 of 5-15 ng/mL is considered appropriate[4]. Increased hematological toxicity and hyperlipidemia in human were observed when SRL C_0 exceeded 15 ng/mL[7].

In 1995, consensus guidelines for TDM of SRL were developed[11]. Much has changed since the original consensus document[3]. Whole-blood trough levels are indicated for TDM[3,7,11,21]. The recommended time for collection is within 1 hr prior to the next oral dose[11]. For multiple daily dosing, collection should be made at the same time during the day to ensure intrapatient consistency[11]. The frequency of monitoring of SRL is lower than CNI due to its long half-life[3,22]. A new steady state can be achieved one week or longer after dosage initiation or adjustment.

Measurement of SRL blood concentration is not a requirement of the U.S. FDA, but Europe health authorities[3]. TDM is recommended for pediatric patients and those with hepatic impairment even when fixed-dose regimen is used. In SRL-based regimen or concentration-guided dosing, routine TDM of SRL is needed[3]. TDM is also recommended when potent inducers or inhibitors of CYP3A4 are co-administered, or when dose of concurrent cyclosporine is markedly reduced or discontinued[3,11].

II. *Chemical Properties and Sample Collection of SRL*

SRL is a white to off-white powder, insoluble in water; very slightly soluble in hexane and petroleum ether; soluble in methanol, diethyl ether, and *N*, *N*-dimethylformamide (DMF); and freely soluble in benzyl alcohol, chloroform, acetone, and CH$_3$CN[23,24]. As 95% of SRL is partitioned into red blood cells, whole blood is the established biologic matrix for routine TDM[3]. Human whole-blood samples should be collected in potassium ethylenediamine-tetraacetate (EDTA) containing blood-collection tubes and protected from light by wrapping in foil or brown plastic bag[25]. Samples collected in this fashion are stable for up to one week at 2-8°C, and up to 3 months at -20°C. For prolonged storage of specimens, -70°C or -80°C is recommended. Although stability of SRL in blood samples has been shown at temperatures up to 30°C[26], SRL in blood samples is unstable at temperatures above 35°C[27].

III. *HPLC-UV Assays for SRL*

Assay of SRL by HPLC-UV is a challenge to many

laboratories[27-29]. Several validated HPLC-UV assays have been reported for determining SRL concentration in whole blood (Table 4)[12-18]. The absolute analytic recovery of an HPLC method used to measure SRL should be >60% with the analytic recovery relative to an IS in the range of 90-110%[11]. The absolute recovery rates reported by Yatscoff *et al.*[15] and Svensson *et al.*[12] were not satisfactory, whereas their relative recoveries met the requirement (Table 4). Results for a new procedure should be compared with a validated one for at least 30 specimens of blood per transplant type or 100 in total. Standard statistical evaluation of the linear-regression data should be studied, including slope and standard deviation (SD) of the slope, y-intercept, SD of the intercept, and SD of the estimate (Sy/x, standard error (SE) of the regression). *t*-test should be performed on the slope and the intercept to demonstrate that they are not statistically different from 1.0 or 0.0, respectively at the $CI_{95\%}$. Slope should differ by <10% from the line of identity (slope 1.0: acceptable 0.9-1.1), the y-intercept, and the Sy/x by no more than 5 ng/mL [11]. Proficiency test is required in Europe[3]. The laboratory must analyze a set of 85-100 samples to test their inaccuracy, repeatability & reproducibility for the measurement of SRL. In addition, three blindness samples need to be analyzed each month to demonstrate their continued proficiency, including 2 spiked to a known concentration, and another pooled sample from patients receiving SRL.

Interfering peaks in the chromatograms, requiring tedious extraction procedures and long run times to resolve the peaks, complicated most of these assays. A limit of quantification (LOQ) of 2.5 ng/mL or lower is important as SRL C_0 of most renal transplant recipients has been maintained at 5-15 ng/mL[8].

Most of these methods required a sample volume of $\leqq$ 1 mL, adopted 32- desmethoxy rapamycin as the IS, and used methanol or methanol water to prepare stock standards of SRL and the IS (Table 4).

IV. *Extraction of SRL from Whole Blood*

One of the most common problems associated with SRL HPLC-UV assays of samples from biologic matrices is chromatographic interference. Rigorous and technically demanding extraction procedures are required to remove proteinaceous and other substances that strongly absorb UV light at 280 nm, a wavelength close to the relatively weak UV extinction maximum of SRL (278 nm)[25]. The method reported by French *et al.*[18] had proteinaceous substances precipitated by $ZnSO_4$ as those required by HPLC/MS/MS methods and made the following extraction procedure easier.

Liquid/liquid extraction is universally used, whereas solid phase extraction was used by Svensson *et al.*[12] only. Only glass tubes should be used in the extraction procedure. Polypropylene tubes used during the extraction may cause interferences. Teflon-coated screw caps or phenolic caps assembled with Teflon liners are also crucial during the

extraction procedure[13,14]. Cardboard seals interfere with chromatography[16]. All the methods add alkali or buffer to samples before extraction with organic solvent (Table 4). When extraction was performed at high pH (e.g. adding Na_2CO_3, or K_2CO_3), SRL and especially the IS isomerized. The isomerization occurred to a lesser extent without addition of alkaline buffer, and was negligible at pH 4.7 (Na acetate buffer)[12]. However, our study using method reported by French *et al.* revealed that addition of adequate alkali was crucial during the extraction of SRL before HPLC-UV assay. For months, the assay outcome was too unstable to achieve a calibration curve. The assay could be continued only after increasing the volume of NaOH. As the most stable range of pH for SRL in aqueous solution is between pH 4.5 and 6.0, it is safe as long as the pH does not exceed 6.0-6.5 during this extraction step. With inadequate alkali, the recovery of SRL and the IS may drop dramatically (Figure 2 (A), (B), (C) and (D)).

Most procedures utilized 1-chlorobutane (butyl chloride) as extraction solvent (Table 4). Svensson *et al.*[12] indicated that extraction with 1-chlorobutane gave a clean extract but low recovery of SRL and the IS. Diethylether gave higher recovery but dirty extract with too much residue after evaporation; while a mixture of equal amounts of 1-chlorobutane and ether gave a higher recovery than pure 1-chlorobutane and a relatively clean extract. Due to possible interference from extraction solvent of some brands, it is important to have extraction solvent tested before starting the procedure.

As 95% of SRL is partitioned into red blood cells, vortex or shaking is very important for adequate and uniform recovery. Extraction time varied in different methods. By using reciprocal shaker, Svensson *et al.*[12] indicated that 30 min of extraction gave higher recovery than 15 min, whereas 60 min extraction did not give any further improvement. In our experience, vortex time longer than 2 min may be required if there is no further shaking. Temperature used during evaporation of the extract ranged from room temperature to 60°C (Table 4). Although SRL is heat unstable, it is important to keep temperature at 25°C or higher during evaporation, especially during winter.

A further purification with reverse-phase solid-phase extraction may give clear colorless solution if the residue from the liquid/liquid extraction was not clean enough for injection on HPLC[12]. Back extraction with hexane also reduced UV interferences[15,16,18].

Because of the light-sensitivity of SRL, early studies required that all work performed in a darkened fume hood[13,14]. However, such requirement did not appeared in the latter study[18].

V. *HPLC-UV Condition for SRL Assay*

The maximal absorbance by SRL at 276 or 278 nm was used in UV detection. In some HPLC systems, SRL and the IS exhibit two peaks in the chromatogram[12,18]. In solution these compounds exist as an equilibrium mixture

of rapidly inter-converting isomers A, B, and C. The major component of SRL is isomer B, while isomer C accounts for approximately 3-10% of various SRL formulations. The amount of isomer A in the formulation is generally < 0.5% (Information from Wyeth-Ayerst).
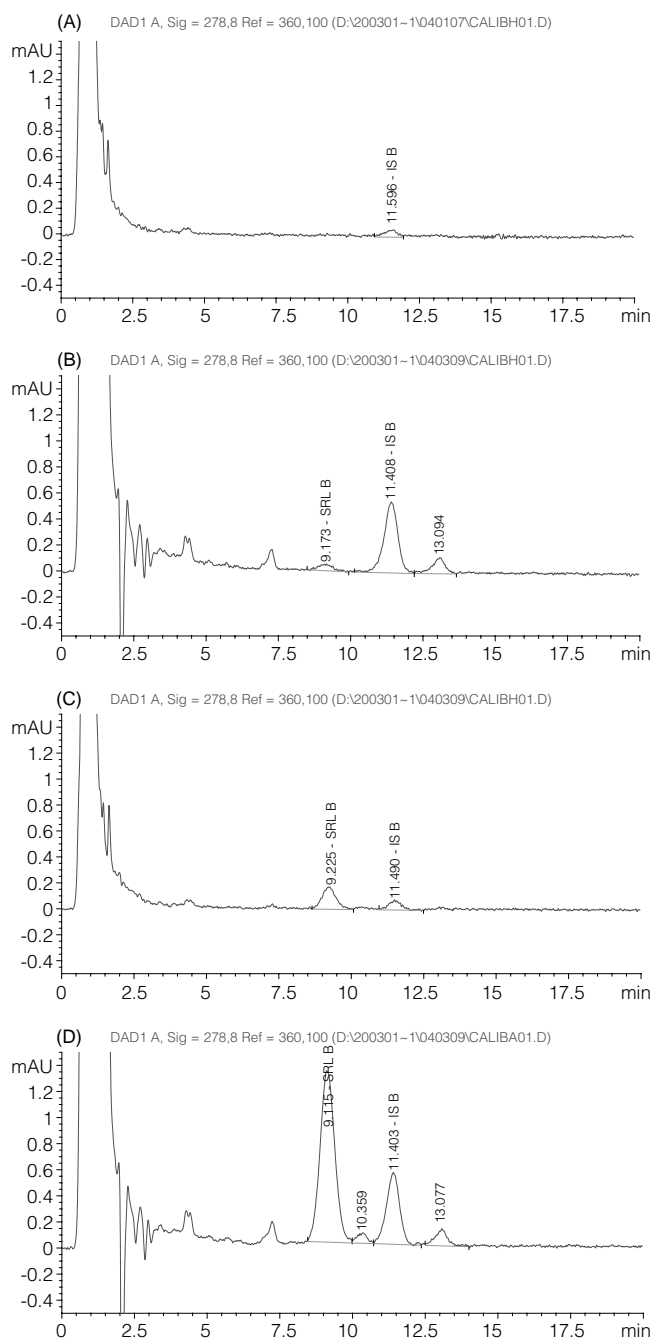

Figure 2. Chromatograms of (A) extract of blank whole blood spiked with 2.5 ng/mL SRL and 32.6 ng/mL IS, and alkalized with 200 µL of NaOH, (B) extract of blank whole blood spiked with 2.5 ng/mL SRL and 32.6 ng/mL IS, and alkalized with 300 µL of NaOH, (C) extract of blank whole blood spiked with 75 ng/mL SRL and 32.6 ng/mL IS, and alkalized with 200 µL of NaOH, and (D) extract of blank whole blood spiked with 75 ng/mL SRL and 32.6 ng/mL IS, and alkalized with 300 µL of NaOH.

Mobile phase played a vital role in the HPLC/UV analysis of SRL. Our preliminary study revealed that CH_3CN 60% in H_2O, as that adopted by French *et al.*[18], could successfully separate isomer B and isomer C of SRL and the IS. But the mobile phase $MeOH/CH_3CN/H_2O$ (68/2/30 in volume as reported by Maleki *et al.*[16] or 68/12/20 in volume) failed to elute either SRL or the IS within 1 hr if Inertsil OD3 column was used. After changing the mobile phase to $MeOH/CH_3CN/H_2O$ (78/2/20 in volume or 73/2/25 in volume), both SRL and the IS were eluted before 17 min, but failed to separate isomer B and isomer C of SRL and the IS. SRL and the IS were not separated either.

Immunosuppressants that were commonly combined with SRL, such as tacrolimus and cyclosporine, and the breakdown product of SRL (e.g., seco-rapamycin), did not interfere with HPLC-UV assay[12].

Reverse-phase C18 column was adopted by various methods. A precolumn filter (guard column, column saver) was often used. The injected volume ranged from 20 µL to 200 µL. The most frequently used mobile phase was methanol water, CH_3CN water, or mixture of methanol, CH3CN and water (Table 4).

Peak-height ratio of SRL/IS was used for SRL quantification. Flow rate in different methods ranged from 0.3 mL/min to 1.5 mL/min. Column temperature employed by different HPLC-UV methods ranged from 40-70°C. HPLC-UV at room temperature at a commonly flow rate resulted in very broad peaks. Svensson *et al.*[12] indicated efficacy can be improved by increasing column temperature to 70°C, and decreasing flow rate to 0.3 mL/min. From our experience, a column temperature slightly below the recommended 50°C resulted in unstable outcomes, especially in LOQ (Table 5). It is important to keep column covered and away from windway of air conditioner to maintain consistent temperature (50°C). In addition, an autosampler temperature at 4 ± 2°C is required to maintain the stability of SRL in reconstituted solution. However, our experience showed that rising autosampler temperature to 6°C during winter may give more consistent results.

Table 5. Influence of column temperature on the outcome (N=10 runs)

Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Recovery (%)	Intraday % CV
2.5	0.0-3.5	0.0-142.9	22.7-141.4%
7.5	4.5 and 9.5	59.9 and 127.2	50.8%
22.5	20.2 and 29.9	89.6 and 132.8	27.5%
58	40.4 and 65.3	69.7 and 112.5%	33.2%

When HPLC was placed under the windway of air conditioner, measured concentrations at LOQ did not meet the requirement of intraday % CV less than 20% in 8 out of 10 runs, even when column oven was covered. On the other hand, only 1 out of 10 did not meet the requirement of intraday % CV less than 15% at higher concentrations.

CONCLUSIONS

The HPLC-UV method we used to determine SRL concentration in human whole blood was validated. SRL whole blood concentration determined by HPLC-UV had a LOQ close to the lower limit of therapeutic range. Samples should be refrigerated and protected from light once collected. Rigorous and technically demanding extraction procedures are required. Extraction solvent should be tested for interference before use. Adequate alkali during extraction, appropriate temperature during evaporation, and sufficient column temperature are key factors of the assay.

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