HPLC Assay with Ultraviolet Detection for Therapeutic Drug Monitoring of Sirolimus, Daniel C. French,1 Michael Saltzgueber,1 David R. Hicks,1* Annabel L. Cowper,2 and David W. Holt 2
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Sirolimus (Rapamune®; rapamycin) is a macrocyclic lactone produced by Streptomyces hygroscopicus (1). It has shown to be effective in animal models of transplant and human clinical trials (6–9) have shown that sirolimus is a potent immunosuppressive agent. Sirolimus is metabolized in humans by hepatic and intestinal cytochrome P450 3A4, primarily leading to demethylated and hydroxylated metabolites (10). When sirolimus is given in the presence of cytochrome P450 3A4 inducers or inhibitors or to patients with hepatic insufficiency, sirolimus blood concentrations may be affected and dose adjustments may be required. Therefore, therapeutic drug monitoring (TDM) of sirolimus concentrations plays an important role in the selection of the optimum dose of sirolimus; in Europe, the license for this drug stipulates that concentrations must be monitored.

Sirolimus binds to the immunophilin FK506 binding protein and is sequestered in red blood cells, leading to whole-blood/plasma ratios of ~38 (11, 12). Because of low sirolimus concentrations in plasma and limited stability in that matrix, whole blood is the matrix of choice for determining sirolimus concentrations (13, 14). An excellent correlation has been reported between sirolimus 24-h whole-blood trough concentrations (cmin) and sirolimus area under the concentration–time curve (12, 15). A strong correlation between sirolimus cmin values and the evidence of efficacy and adverse events has also been reported (15). Therefore, the TDM of sirolimus cmin values provides a useful prediction of inadequate immunosuppression or potential adverse events. When sirolimus is given with full-dose cyclosporine, the recommended therapeutic range for sirolimus whole-blood trough concentrations is 5–15 µg/L (15). For an analytical method to be suitable for the TDM of sirolimus, it should be simple, sensitive (lower limit of quantification, <5 µg/L), and rapid (turn-around time, <24 h).

Several HPLC assays using ultraviolet (UV) detection (HPLC-UV) have been reported for the determination of sirolimus concentrations in whole blood (16–20). The assays are complicated by interfering peaks in the chromatograms, requiring tedious extraction procedures and long run times to resolve the peaks. The goal of the present study was to develop a simple HPLC-UV assay for sirolimus that was precise and accurate at low concentrations and was capable of a high throughput. To accomplish this, it was necessary to eliminate interfering peaks at the sirolimus retention time and to decrease the run time by eliminating late-eluting peaks. Here we describe a simple and rapid HPLC-UV method for the determination of sirolimus concentrations in human whole blood that meets these requirements. The validation was performed in accordance with regulatory guidelines (21).

Sirolimus was obtained from Wyeth-Ayerst Analytical R & D. The internal standard ([IS]; 32-desmethoxy rapamycin) was obtained from Wyeth-Ayerst Research Compound Room. Solvents (water, acetonitrile, acetone, hexane, 1-chlorobutane, methanol; HPLC grade or better) used in the extraction or analytical procedures were purchased from EM Science. Zinc sulfate heptahydrate (GR reagent; 99.5% minimum purity) and sodium hydroxide (500 g/L) were also purchased from EM Science.

Calibrators and quality-control (QC) samples were prepared in EDTA anticoagulated human whole blood (Biological Specialty Corp.), using stock solutions prepared in methanol at a concentration of 1000 mg/L. The stock solutions were prepared in glass volumetric flasks (VWR Scientific Products), transferred to polypropylene tubes (Corning Inc.), and stored at ~80 °C. The calibration curve was based on eight nonzero sirolimus calibrators (2.5, 5, 10, 15, 25, 50, 70, and 75 µg/L). Three internal QC samples (7.5, 22.5, and 58 µg/L) were prepared from an independent stock solution. Calibrators and QCs were transferred to polypropylene tubes (Corning Inc.) and stored frozen at ~80 °C until use. The IS solution was prepared at a concentration of 250 µg/L in 500 mL/L methanol in water and stored in propylene tubes (Corning Inc.) at ~80 °C until used.

The whole-blood sample (0.5 mL) and IS (75 µL) were transferred to a 13 × 100 mm screw-capped tube (Teflon®-lined cap; Corning Inc.) and vortex-mixed well. Zinc sulfate solution (1 mL; 50 g/L) and 1 mL of acetone were added to each tube. The tubes were capped, vortex-mixed (Multitube vortexer; Baxter Diagnostics, Inc.) for 20 s, and centrifuged (Sorvall RC3C Plus) at 2600g and ambient temperature for 5 min. The supernatant was transferred to a clean 13 × 100 mm tube. NaOH (200 µL; 100 mmol/L) was added to each tube, followed by vortex-mixing, 1-Chlorobutane (2 mL) was added to each sample tube and the tubes were capped, vortex-mixed for 1 min, and centrifuged at 2600g and ambient temperature for 5 min. The supernatant was transferred to a clean 10-mL conical centrifuge tube (Kimble Glass, Inc.). The supernatant was
Fig. 1. HPLC chromatograms of extracts of control human whole blood (A), control human whole blood supplemented with 7.5 μg/L sirolimus (B), and whole blood from a patient receiving Rapamune (C; sirolimus concentration, 4.3 μg/L). 1, sirolimus; 2, IS.
dried under nitrogen (TurboVap; Zymark) at ambient temperature for 30 min. The dried extracts were reconstituted with mobile phase (150 μL), and then hexane (500 μL) was added to each tube. The tubes were then capped, vortex-mixed for 30 s, and centrifuged at 2600g and ambient temperature for 2 min. The hexane layer was removed from each sample and discarded. The extracts were briefly dried under nitrogen (TurboVap, Zymark) at ambient temperature for 1 min to remove any remaining hexane. The extracts were transferred to limited volume autosampler vials (350-μL Macrovials; Scientific Resources, Inc.). The vials were capped with Teflon-lined snap-caps (Scientific Resources, Inc.) and placed on the HPLC autosampler, which was set to inject 50 μL.

The HPLC system consisted of the following: an analytical column (Alltech C18 with 5-μm beads; 150 mm × 2.1 mm; Alltech); an autosampler (model AS3000; ThermoSeparation Products) equipped with a column oven set to 50 °C; a pump (model ConstaMetric 4100; ThermoSeparation Products) set to deliver 0.5 mL/min; an UV detector (model UV 2000; ThermoSeparation Products) set at 278 nm (range, 0.01 absorbance units, full scale; rise time 1 s); and a data acquisition system (PE Nelson Turbochrom, Ver. 6.1). The mobile phase (600 mL/L acetonitrile in H2O) was pumped at 0.5 mL/min, which led to a typical back pressure of 900 psi. Sirolimus and the IS were detected by UV absorption at 278 nm at retention times of ~9 and ~11 min, respectively. Sirolimus concentrations (μg/L) were obtained from the regression line relating the peak-height ratios of sirolimus/IS to the sirolimus concentrations (μg/L). These were calculated using an eight-point calibration curve and weighted linear regression, with weight = 1/concentration.

The selectivity of the method was demonstrated by the absence of any detectable extraneous chromatographic peaks with retention times equal to those of sirolimus or the IS in the extracts of control human whole-blood samples from 15 different volunteers. Representative chromatograms for extracts from a control human whole-blood sample, a low QC sample (7.50 μg/L), and a sample from a patient receiving sirolimus (4.3 μg/L) are presented in Fig. 1, A, B, and C, respectively.

The lower limit of quantification was assessed by calculating the imprecision and accuracy for replicate samples (n = 5) at the concentration of the lowest calibrator. The CV and bias were 10% and −1.8%, respectively. The upper limit of quantification was set at the value of the highest calibrator: the corresponding CV and bias were 5.3% and −2.5%, respectively. The intra- and interday CVs and the accuracy of the method were assessed by calculating daily and overall CV and bias values for QC sample sets (five replicates of each concentration per analytical run) that were assayed in four analytical runs. The intra- and interday imprecision CVs were 0.9–8.5% and 2.5–6.9%, respectively; and the intra- and interday bias values were −3.7 to 7.5% and 1.1–5.2%, respectively (Table 1).

The recoveries of sirolimus and the IS were determined by measuring the peak heights of sirolimus and the IS in extracted blood samples and comparing them with those obtained from unextracted calibrators. QC sample sets (five replicates of each concentration) were extracted and peak heights were compared with unextracted samples (five replicates of each concentration) prepared in mobile phase. The mean recoveries of sirolimus and the IS in human whole blood were 80.6% and 81.8%, respectively. The stability of sirolimus in human whole blood after three freeze–thaw cycles and after storage at ambient temperature has been reported previously (19, 20).

To demonstrate that an unfrozen sample can be successfully extracted and read off a frozen calibration curve, QC samples (five replicates/concentration) were prepared in fresh unfrozen blood at concentrations of 7.5, 22.5, and 58 μg/L and extracted along with frozen QC samples at the same concentrations. The values obtained for the unfrozen samples (7.34 ± 0.81, 22.1 ± 1.12, and 53.2 ± 4.56 μg/L) were not significantly different from the values obtained for frozen samples (7.20 ± 0.77, 22.6 ± 1.49, 55.5 ± 2.44 μg/L). The data indicate that unfrozen samples can be read off a frozen calibration curve.

Sirolimus concentrations in 86 whole-blood trough samples from renal transplant patients were determined by the present method and compared with those obtained by the HPLC method using tandem mass spectrometric detection described by Holt et al. (22). There was an excellent agreement between the sirolimus whole-blood concentrations obtained by the two methods. Correlation of the paired values by linear regression produced a correlation coefficient of 0.9765, a slope of 0.9844 ± 0.0237, and a y-axis intercept of 0.0777 ± 0.2341.

In a further study to document assay performance, 78 blinded samples, packaged as five batches of samples, were analyzed. These samples are currently sent to laboratories establishing an assay for sirolimus, to test their proficiency for the measurement (23). Three batches of 17 samples contained 5 aliquots each of three samples supplemented with sirolimus (nominal concentrations, 7, 15, and 25 μg/L), plus 1 aliquot of an out-of-range sample (nominal concentration, 200 μg/L) and 1 aliquot of a sirolimus-free sample. An additional batch contained 15 samples comprising 5 aliquots of 3 pooled samples from patients receiving sirolimus, whereas the fifth batch contained 5 nonzero sirolimus calibrators in duplicate and a sirolimus-free, whole-blood sample in duplicate. Within each batch, the samples were randomized. The first three batches were analyzed on separate working days, whereas the final batches were analyzed in a single

| Table 1. Imprecision and accuracy for the determination of sirolimus concentrations in whole blood. |
|------------------|---------------|---------------|---------------|---------------|
|                  | Intraday      | Interday      |
| Sample (μg/L)    | CV, %         | Bias, %       | CV, %         | Bias, %       |
| LLOQ (2.5)       | 10            | −1.8          |               |               |
| Low QC (7.5)     | 8.5           | 6.4           | 6.9           | 1.1           |
| Mid QC (22.5)    | 1.8           | 5.2           | 2.5           | 3.7           |
| High QC (58)     | 1.6           | 6.3           | 2.9           | 5.2           |
working day, in two separate assays. The out-of-range samples were reanalyzed on the final day, after appropriate dilution.

The within- and between-batch CVs for the control samples were <10% and the within-batch CV for the patient samples was <10%. The inaccuracy, against a value defined by HPLC–tandem mass spectrometric measurement using independently prepared calibrators (19), was <6% for the control samples and <13% for the three pooled patient samples (defined sirolimus concentrations were 8.3, 15.7, and 28.4 μg/L). The sirolimus-free samples were identified correctly and the inaccuracy for the out-of-range sample was 6.7%.

In summary, a HPLC-UV method for the quantification of sirolimus in human whole blood has been developed and validated. With the run time (<15 min) and the simplified extraction procedure described, 48 samples and the requisite calibrators and QC samples can be extracted and quantified in <20 h. The assay is linear over the range of 2.5–75 μg/L, based on a 0.5-mL sample. The data presented in this report demonstrate that the method provides rapid, sensitive, precise, and accurate measurements of sirolimus concentrations in human whole blood.

References


Determination of D-Mannose in Serum by Capillary Electrophoresis, Hubert A. Carillon and Jaak Jaeken (Centre for Metabolic Disease, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium; * address correspondence to this author at: Centre for Metabolic Disease, Campus Gasthuisberg O&N, Herestraat 49, B-3000 Leuven, Belgium; fax 32-16-347284, e-mail hubert.carillon@med.kuleuven.ac.be)

Congenital disorders of glycosylation (CDG) are a newly delineated group of inherited multisystem disorders associated with abnormal glycosylation of glycoproteins (1). In CDG group I, which includes all defects in N-glycan assembly (2), the subgroup CDG type Ib, attributable to phosphomannose isomerase (EC 5.3.1.8.) deficiency, is treatable by mannose supplementation (3). Monitoring of this treatment necessitates the availability of methods to quantify D-mannose in serum. The determination of mannose in serum is hampered by the presence of an ~100-fold excess of glucose. Jolley et al. (4) used high-resolution liquid chromatography, whereas Aloia (5) used gas-liquid chromatography after treating the sera with glucose oxidase. Soyama (6) and Akazawa et al. (7) used enzymatic methods that involved treatment with glucose oxidase. In all of these studies, the presence or elimination of glucose remained critical. Pitkänen and Kanninen (8) were able to measure mannose using gas chromatography–mass spectrometry. However, this method is not suitable for routine purposes. The assay proposed by Etchison and Freeze (9) involves the elimination of glucose by glucokinase (EC 2.7.1.2), followed by the removal of anionic products by a subtle ion-exchange chromatography step. Finally, the mannose concentration is determined enzymatically.

We investigated whether capillary electrophoresis (CE) of fluorophore-labeled carbohydrates was an appropriate method. In the resulting procedure, D-mannose can be determined in small amounts of serum in the presence of...