Validation of an Assay for Routine Monitoring of Sirolimus Using HPLC with Mass Spectrometric Detection, David W. Holt,1* Terry Lee,1 Kirsty Jones,1 and Atholl Johnston2 (1 St. George’s Hospital Medical School, London SW17 ORE, United Kingdom; 2 St. Bartholomew’s & The Royal London School of Medicine & Dentistry, London EC1M 6BQ, United Kingdom; * address correspondence to this author at: Analytical Unit, St. George’s Hospital Medical School, London SW17 0RE, UK; fax 44-20-8767-9687, e-mail d.holt@sghms.ac.uk)

Sirolimus (rapamycin, Rapamune®; Wyeth-Ayerst Research) is a potent immunosuppressive drug with a molecular mass of 913.6 Da (1). When licensed in the United States, measurement of circulating sirolimus concentrations was recommended for pediatric patients, for those with hepatic impairment, and for patients in whom potent inducers or inhibitors of the enzyme CYP3A4 are co-administered or in whom concomitantly administered cyclosporine dosing is markedly reduced or discontinued. Because 95% of the drug is sequestered within erythrocytes, whole blood has been recommended as the sample matrix (2).

In a series of concentration-controlled clinical trials, sirolimus was measured by HPLC with mass spectrometric (MS) detection (3) or HPLC with ultraviolet (UV) detection as a guide to dose adjustment (4, 5). For the double-blind pivotal phase III clinical trials, the drug was measured by an investigational microparticle enzyme immunoassay (Abbott Diagnostics) on the IMx® clinical analyzer (6). The immunoassay is not commercially available, and there is an acute need for an accurate, rapid, and simple chromatographic assay to manage patient care. Current clinical trials target predose (trough) whole-blood sirolimus concentrations within the approximate range 5–30 μg/L.

HPLC-UV assays for sirolimus have throughputs of only ~20–30 samples per day, and current sirolimus dosage regimes tend to achieve sirolimus trough concentrations close to the reported lower limits of quantification (LLOQ) of these assays, ~1–5 μg/L, despite the use of blood sample volumes in the range 1–2 mL (7–9). For most laboratories, the only feasible alternative to HPLC-UV currently available is HPLC-MS (10, 11), which has a shorter chromatographic run time (leading to a higher sample throughput), smaller sample volume, increased selectivity for sirolimus, and a lower LLOQ. With sample volumes of 0.5 or 1 mL, the LLOQ is 0.2 or 0.25 μg/L, respectively (10, 11). We describe an HPLC-MS assay designed to measure up to 100 samples per day. The validation was performed in compliance with Good Laboratory Practice and regulatory guidelines (12, 13).

The aim of the validation was to establish the following parameters: within- and between-assay reproducibility, linearity, accuracy, recovery of sirolimus from whole blood, selectivity in the presence of sirolimus metabolites, and demonstration that matrix effects did not lead to ion suppression. The stability of whole blood samples containing sirolimus at room temperature, after repeated freezing and thawing, and under conditions likely to be encountered during transport of study samples to a central laboratory were also studied. In addition, the stability of the sample extract was assessed over a period of 24 h, and the results of this assay for sirolimus in patient samples were compared with those obtained by the immunoassay.

Sirolimus, nor-rapamycin (internal standard), and sirolimus metabolites1 1 (hydroxylated), 3 (hydroxylated, 7-O-demethylated), 6 (6-hydroxylated), 8 (seco-rapamycin), and 9 (41-O-demethylated) were supplied by Wyeth-Ayerst Research (Princeton, NJ), together with EDTA-anticoagulated sirolimus-free human whole blood. HPLC-grade methanol was obtained from Rathburn Chemicals Limited. Zinc sulfate, sodium hydroxide, ammonium acetate, ethanol, ethanediol, 1-chlorobutane, and acetone were purchased from Merck (BDH). Deionized water was prepared on site (ELGA Limited).

Calibrators and in-house control samples were prepared in EDTA-anticoagulated sirolimus-free human whole blood, using separate stock solutions prepared in methanol. Six non-zero calibrators (nominal values of 1, 2.5, 5, 10, 25, and 50 μg/L) and three in-house control samples [nominal values of 3.0, 15.0, and 30.0 μg/L (low, medium, and high)] were prepared, together with an out-of-range control sample with a nominal value of 199 μg/L. Calibrators and controls were aliquoted and stored at approximately −20 °C before use.

For sample preparation, 100 μL of calibrators, quality-control samples, or patient samples; 100 μL of internal standard [dissolved in ethanol-ethanediol-water (50:25:25, by volume)]; 500 μL of 50 g/L zinc sulfate solution; and 500 μL of acetone were pipetted into 2-mL polypropylene tubes, which were mixed for 5 min [IKA-Vibrax-VXR mixer; Merck (BDH)] and then centrifuged at ~12 000g for ~5 min. The solvent layers were decanted into 4.5-mL polypropylene tubes with 100 μL of 0.1 mol/L sodium hydroxide and 2 mL of 1-chlorobutane. After mixing for 5 min, the tubes were centrifuged at ~2000g for ~5 min. The solvent layers were then transferred to 4.5-mL conical polypropylene tubes, placed in a Savant SpeedVac® (ThermoQuest) at 60 °C, and evaporated to dryness. The dried extracts were reconstituted with 800 mL/L methanol (250 μL), mixed for 5 min, and then transferred to autosampler tubes, which were placed in an autosampler tray set at ambient temperature. The autosampler injected a 100-μL aliquot of each extract onto the analytical column.

The analytical column was a 15 cm × 4.6 mm (i.d.) Supelcosil LC-18-DB (5 μm particle size) ODS column fitted with a Newguard C-18 precolumn (Sigma-Aldrich), which was maintained at 50 °C with a Perkin-Elmer series 200 column oven (PE Biosystems). Isocratic solvent delivery was achieved using a Perkin-Elmer series 200 pump set at 1 mL/min. Sample injection was performed by a

1 Some metabolites were not isolated in sufficient quantity to obtain more precise information on the exact position of structural modification.
Perkin-Elmer series 200 auto injector fitted with a 200-μL sample loop. The mobile phase consisted of methanol-water (80:20, by volume) supplemented with ammonium acetate solution to achieve a final concentration of 2 mmol/L.

Detection was by tandem mass spectrometry (HPLC/MS/MS), using a Sciex API2000 triple quadrupole mass spectrometer (PE Biosystems). A turbo-ion spray (heated electrospray) source heated to 300 °C was used to introduce the sample into the mass spectrometer. A post-column splitter (10:1) was installed just before the ion spray interface. We used high-purity air as the nebulizer gas, high-purity nitrogen as the collision gas, and multiple reaction mode, run in positive ionization mode. The first quadrupole (Q1) was set to detect the ammonium adduct ions [M+NH₄⁺] of sirolimus (m/z 931.8 ± 1) and nor-rapamycin (m/z 917.5 ± 1). The second quadrupole (Q2) was used as the collision chamber. The third quadrupole (Q3) was set to detect the product ions of sirolimus (m/z 864.5 ± 0.5) and nor-rapamycin (m/z 850.0 ± 0.5).

A PowerMac PC running PE Sciex Sample Control (Ver. 1.4) software was used to control the HPLC/MS/MS and record the output from the detector. Integration of peak areas, calculation of peak area ratios, calculation of the slope of the calibration line, and calculation of the sirolimus sample concentrations were performed using PE Sciex TurboQuan™ (Ver. 1.0) software. A 1/x² weighted regression was used to fit the calibration data, with a through-zero option.

The elution times for sirolimus and the internal standard were 6.0 and 5.3 min, respectively. Fig. 1 shows typical chromatograms for extracts from a calibrator and patient sample.

For in-house control samples, measured values were within 3.7%, −0.3%, and −0.6% of the nominal values for the low, medium, and high control samples, respectively (n = 30 at each concentration). To determine within-assay repeatability, each control sample was extracted six times in one batch. The within-batch imprecision (CV) was 7.3%, 1.9%, and 2.6% for the low, medium, and high control samples, respectively. Subsequently, the three control samples were each extracted six times in four additional batches over 6 working days. For each assay, a separate calibration curve was used. For each of the five assays, the concentration for the first aliquot of each control sample was used to calculate the between-assay reproducibility. The CV for each was 8.1%, 3.8%, and 3.0% (low, medium, and high, respectively). The results for these three control samples were then recalculated using only the first generated calibration curve to test the validity of using a single calibration curve over a period of 1 working week. Results were 1.0%, −2.9%, and −3.0% of control, and overall imprecision (CV) was 7.0%, 3.0%, and 3.0% for the low, medium, and high control samples, respectively. Subsequently, results for consecutive analyses of the low control sample (n = 75), in which six technicians were involved, showed a mean value of 3.1 μg/L (target, 3.0 μg/L) with a CV of 8.6%.

The LLOQ was set at the value of the lowest calibrator (1 μg/L). We assessed the repeatability at this concentration by extracting the lowest calibrator six times in one batch. The percentage deviation from the nominal value was 16%, and the imprecision (CV) was 7.8%. The upper limit of accurate quantification (ULOQ) was set at the value of the highest calibrator, and the comparable data were −2.1% and 3.2%, respectively.

Assay linearity, with a view to dilution of samples producing results above the ULOQ, was assessed using the out-of-range control sample (nominal value, 199 μg/L). This sample was diluted 1:5, 1:10, and 1:25 using sirolimus-free whole blood, and each dilution was assayed three times. The mean percentage deviation from the original nominal value was <5.5% for all dilutions.

The three in-house control samples were used to assess the absolute recovery of sirolimus and the internal standard from human whole blood. The control samples were extracted as described above, and the extracts were injected onto the analytical column. The resulting peak areas were compared with those produced by reference standards, which were prepared in a manner identical to the in-house controls, except that all dilutions were made in 800 mL/L methanol in deionized water. Aliquots (100 μL) of these reference standards were then mixed with 100 μL of the internal standard (100 μg/L), together with 50 μL of 800 mL/L methanol before direct injection onto the analytical column. Each extracted sample was analyzed six times; each reference standard was injected four times. Overall, recoveries of sirolimus and the internal standard averaged 91% and 83%, respectively.

The effect of storage at 4 °C, ambient temperature (nominally 20 °C), and 35 °C was tested using one in-house control sample (medium) and a pooled sample from patients receiving sirolimus (mean concentration, 10.7 μg/L) over a period of 6 days. The samples from patients receiving sirolimus were pooled and analyzed within 30 h of collection for routine monitoring. Each sample was analyzed in triplicate at each time point. There was no trend toward a loss of sirolimus during storage at either 4 or 20 °C. At 35 °C, there was a 9% loss of the drug after 3 days and a 13% loss after 6 days. The effect of repeated freezing (approximately −20 °C) and thawing for three freeze/thaw cycles was tested using the same in-house control and pooled sample used to test temperature stability. There was no trend toward a loss of sirolimus during this procedure.

The sample extract was stable after extraction for 30 h at −20 °C, the temperature of the autosampler rack.

Only metabolite 8 produced initial and product ions similar to the parent compound, but the product ion for the metabolite was well resolved from the product ion for sirolimus, with a retention time of 2.5 min. Metabolite 9 had initial and product ions similar to the internal standard but was almost resolved from the ion of interest. This did not pose a problem, as demonstrated by the chromatography of extracts of samples from patients receiving sirolimus (five from kidney and five from liver transplant patients), without the addition of internal standard. A peak corresponding to metabolite 9 was detected.
in five samples, but the peak area would have contributed, at most, 2% to the peak area of the internal standard because the internal standard was present in large excess compared with the metabolite and the two peaks only partially overlapped.

Absence of ion suppression was demonstrated by the method of Matuszewski et al. (14). A total of 10 EDTA-anticoagulated blood samples from patients receiving either cyclosporine (n = 5) or tacrolimus (n = 5) after kidney transplantation were extracted by the method described above. None of the patients was receiving sirolimus. The final dried extracts were reconstituted with a reference solution of sirolimus and nor-rapamycin in 800 mL/L methanol at concentrations equivalent to a 10 μg/L calibrator. The reconstituted extracts and the reference solution of sirolimus and internal standard were injected onto the analytical column. The peak areas for sirolimus and the internal standard for the extracted samples were compared with the mean of those produced by the reference solution (n = 4 injections). The differences for the peak areas between the extracted samples and the reference solution averaged −1% for sirolimus and 1% for the internal standard (95% confidence intervals, −3.2% to 1.3% and −1.5% to 3.2%, respectively). These findings confirmed that there was no influence of

![Chromatograms](image-url)

Fig. 1. Chromatograms of an extract of a whole-blood sirolimus calibrator with a nominal value of 10 μg/L (A) and an extract of a whole-blood sample from a patient receiving sirolimus (B). (B), the calculated concentration of sirolimus was 4.1 μg/L.
Table 1. Results for HPLC/MS/MS and IMx measurements of sirolimus in 36 proficiency testing samples circulated by the International Sirolimus Proficiency Testing Scheme.

<table>
<thead>
<tr>
<th>Method</th>
<th>Sample</th>
<th>Nominal concentration, µg/L</th>
<th>Difference, %</th>
<th>Median</th>
<th>1st quartile</th>
<th>3rd quartile</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC/MS/MS</td>
<td>Sirolimus added</td>
<td>&lt;10</td>
<td></td>
<td>0</td>
<td>-2.9</td>
<td>12.5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Sirolimus added</td>
<td>10–20</td>
<td></td>
<td>-2.4</td>
<td>-5.0</td>
<td>1.7</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Sirolimus added</td>
<td>&gt;20</td>
<td></td>
<td>2.5</td>
<td>-5.2</td>
<td>4.3</td>
<td>6</td>
</tr>
<tr>
<td>IMx</td>
<td>Sirolimus added</td>
<td>&lt;10</td>
<td></td>
<td>-5.8</td>
<td>-11.0</td>
<td>1.7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Sirolimus added</td>
<td>10–20</td>
<td></td>
<td>-3.2</td>
<td>-7.7</td>
<td>-0.2</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Sirolimus added</td>
<td>&gt;20</td>
<td></td>
<td>-5.5</td>
<td>-9.4</td>
<td>-2.4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Patient pools</td>
<td>10</td>
<td></td>
<td>31.0</td>
<td>18.0</td>
<td>38.0</td>
<td>13</td>
</tr>
</tbody>
</table>

*a* The difference was calculated as difference from the nominal concentration for the samples with added sirolimus and from the HPLC/MS/MS result in the case of the patient pools.

the matrix on the detection of either sirolimus or the internal standard.

Fifty predose samples from 40 kidney transplant recipients were analyzed by immunoassay on the IMx and then stored at approximately −20 °C before analysis by HPLC. The sirolimus concentration was, on average, 30% higher (95% confidence interval, 24–36%) by immunoassay on the IMx than HPLC/MS/MS, consistent with immunochromatographic cross-reactivity of sirolimus metabolites, as documented for hydroxy-rapamycin and 41-O-demethyl-rapamycin (6). The equation for the Passing and Bablok regression line (15) was: IMx = (1.32 × HPLC/MS/MS) − 0.1 (by linear regression, IMx = 1.25× HPLC/MS/MS; r² = 0.84; S/√y = 3.0). Thus, for those laboratories converting from the immunoassay to HPLC/MS/MS, our data would suggest that they might expect an ~20–25% decrease in the measured concentration of sirolimus.

Samples from the first 12 challenges of the International Sirolimus Proficiency Testing Scheme (16) were analyzed by HPLC/MS/MS, comparing the results with the nominal values of samples with added sirolimus and the median value for laboratories using the IMx assay (n = 29–42) for pooled patient samples. Comparisons were made for 21 samples with added sirolimus (nominal concentrations, 3.0–27.5 µg/L) and a sirolimus-free sample, together with 11 pooled samples from patients receiving sirolimus (3 from liver transplant recipients and 8 from kidney transplant recipients). The pooled samples gave results between 7.8 and 12.1 µg/L by HPLC/MS/MS. There was better agreement between the expected value and the result produced by the chromatographic assay (Table 1). The median difference between HPLC/MS/MS and the immunoassay was similar to that noted above but less than that reported recently by others (17). The relative difference between results obtained by chromatography and immunoassay for sirolimus is likely to vary, depending on such factors as hepatic function and sample timing after the last oral dose.

In conclusion, the method described here is rapid, reproducible, selective, and requires only a small sample volume. We have used the method on a daily basis for >3 months at this central laboratory to measure sirolimus in samples from ongoing clinical studies of the drug throughout Europe. Up to 100 samples per day have been analyzed and reported, making centralizing facilities for large clinical studies feasible. The assay has also been suitable for the measurement of sirolimus in blood samples from pediatric patients, for which sample volume was a critical factor. The system has been stable, allowing only weekly calibration, with good quality-control sample performance throughout this period. Because of the small sample volume and the cleanliness of the extracts, the precolumn has lasted through ≥1000 injections. Furthermore, because the flow is split before introduction of the extract into the mass spectrometer, scheduled cleaning of the instrument curtain plate is required only after 2500 or more injections, typically after 6–8 weeks. The LLOQ is more than adequate for monitoring the drug at the concentrations resulting from current dosage regimes. This validation has also confirmed the difference to be expected when a selective chromatographic technique is used to measure sirolimus, compared with the noncommercial, investigational immunoassay. Our data also justify the advisability of transporting samples between study sites and a central laboratory, using shipping at subambient temperatures (16).

References

Identification of Two LDL-Receptor Mutations Causing Familial Hypercholesterolemia in Indian Subjects by a Simplified Rapid PCR-Heteroduplex Method, Tester F. Ashavaid,1 Altaf A. Kondkar,1 and Kappiareth G. Nair2 (1 Research Laboratory, P. D. Hinduja National Hospital & Medical Research Center, Mumbai 400 016, India; 2 Breach Candy Hospital & Research Center, Mumbai 400 026, India; * author for correspondence: fax 91-22-4449151, e-mail dr_tashavaid@hindujahospital.com)

The molecular analysis of familial hypercholesterolemia (FH), an autosomal dominant disease caused by a multitude of LDL receptor (LDLR) gene mutations, is complicated by mutational heterogeneity of the disease in the majority of population studied to date (1). Exceptions occur where the frequencies of specific mutations are increased in a population because of founder effects, or where a mutation has been introduced on many occasions into a small isolated community where a mutation has been introduced on many occasions. The prevalence of FH in India is not known. In addition, to the best of our knowledge there are no published reports on systematic analyses of mutations underlying FH in India. However, to date 10 different LDLR mutations in immigrants from India have been reported in the literature (5). Most of these mutations have been reported in exons 3, 4, 9, and 14 among Indians settled in South Africa, which suggests an increased frequency of FH in India (5). More than 80% of the 150,000 Indians who immigrated to South Africa between 1860 and 1911 originated from diverse areas in India (6). This group has remained isolated as a whole and also within different communities, primarily as a result of religious and cultural practices. This probably indicates that the increased incidence of mutations is attributable to multiple entries of defective LDLR genes into the Indian population of South Africa from India and that the group in South Africa probably represents the incidence in the Indian subcontinent.

The current study was thus undertaken to identify four previously reported LDLR gene point mutations in Indian hypercholesterolemic patients with clinical features of FH. The subjects were screened for mutations W66G (7), E207K (7), E387K (8), and P664L (9) in exons 3, 4, 9, and 14, respectively, which are those most reported among Indians in South Africa. In an attempt to identify other mutations causing FH among Indian subgroups in the same exons by heteroduplex (HDX) analysis, we further modified the technique and applied this approach for rapid detection of mutations in the LDLR gene.

Genomic DNA was extracted from blood samples of an apparently healthy control group and hypercholesterolemic patients with clinical features of FH (10) to detect mutations in exons 3, 4, 9, and 14 of the LDLR gene, using a salting out method (11). The exons were PCR amplified (12) using specific oligonucleotide primers (Table 1) and conditions as described elsewhere (7–9). The known point mutations were detected by restriction enzyme digestion of the amplified DNA as described previously (7–9). HDX analysis was performed with slight modification (13) of the known techniques (14, 15). The amplified products (5 μL) from both controls and patients were mixed and subjected to denaturation and renaturation cycles. Denaturation and renaturation were performed in a programmed PCR thermal cycler (model 480; Perkin-Elmer) at 95°C for 8 min, followed by 1 min each at 90, 80, and 70°C and so forth until room temperature (20°C) was reached. The renatured samples were diluted with equal volumes of gel loading buffer (950 mL/L formamide, 20 mmol/L EDTA, 0.5 g/L xylene cyanol, and 0.5 g/L bromphenol blue) and electrophoresed through low cross-linking 10% denaturing polyacrylamide gels (160 × 180 × 1 mm) supplemented with 75 g/L urea. The low cross-linking (1%) polyacrylamide gel was made from 40% acrylamide solution containing 40% acrylamide and 0.4% N,N′-methylenebisacrylamide. Electrophoresis was performed with 1× Tris-borate-EDTA buffer at 200 V and 23 mA for 3–5 h at room temperature, stained with 0.5 mg/L ethidium bromide in 0.6× Tris-borate-EDTA buffer for 30 min, and visualized under ultraviolet light.

<table>
<thead>
<tr>
<th>Table 1. Primers for PCR amplification.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Region</strong></td>
</tr>
<tr>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>exon 3</td>
</tr>
<tr>
<td>exon 4</td>
</tr>
<tr>
<td>exon 9</td>
</tr>
<tr>
<td>exon 14</td>
</tr>
</tbody>
</table>

*Sense.  
**Antisense.*