Evaluation of a Deuterium-Labeled Internal Standard for the Measurement of Sirolimus by High-Throughput HPLC Electrospray Ionization Tandem Mass Spectrometry

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BACKGROUND: Matrix effects in HPLC–electrospray ionization–tandem mass spectrometry (HPLC-ESI-MS/MS) can cause differences in the ionization of an internal standard (IS) compared with the analyte of interest. Unless sample cleanup or chromatographic conditions eliminate or minimize ion suppression or enhancement, variability in interpatient matrices may cause erroneous results. A stable isotope-labeled IS can be used to minimize analytical interpatient variation.

METHODS: We used protein precipitation and HPLC-ESI-MS/MS to quantify sirolimus (SIR) with both desmethoxyrapamycin (DMR) and deuterium-labeled sirolimus (SIR-d3) as the IS to analyze a range of whole-blood and extraction-matrix samples, and to estimate recovery, matrix effects, process efficiency, and interpatient variation. We also analyzed a series of blood samples from 72 patients taking SIR, including external proficiency-testing samples, with these ISs.

RESULTS: The range of interpatient assay imprecision (CV) values for the SIR assay was consistently lower with SIR-d3 (2.7%–5.7%) than with DMR (7.6%–9.7%). The results obtained with the 2 different ISs for the patient samples showed a linear relationship, but the results were higher with DMR as the IS than with SIR-d3.

CONCLUSIONS: The use of SIR-d3 as the IS in the high-throughput HPLC-ESI-MS/MS assay of SIR yielded improved results compared with the use of DMR. SIR-d3 appears to be less affected by differences in the ionization of SIR and its IS caused by the variability of interpatient matrices. The IS-related difference in SIR estimation needs further investigation.

Matrix effects on electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis have been well documented (1–3). Unless sample cleanup or chromatographic conditions minimize ion suppression or enhancement, variation in interpatient matrices has the potential to cause erroneous results. High-throughput “rapid analysis” methods commonly used for analyzing immunosuppressants and other drugs by HPLC-MS/MS can be adversely affected by limited chromatographic retention and by limited separation of analytes from co-eluting interfering substances (4).

The preferred choices for an internal standard (IS) are (a) versions of the analyte labeled with a stable isotope, (b) analogs of the analyte, or (c) another related molecule that closely matches the chromatographic and ionization characteristics of the analyte of interest (5, 6). Until recently, the lack of isotope-labeled versions of many commonly analyzed therapeutic drugs required the use of a “best alternative” IS.

Our laboratory routinely analyzes sirolimus (SIR) with a high-throughput HPLC-ESI-MS/MS assay modified from that described by Wallemacq et al. (7). In brief, 40 μL of 0.1 mol/L ZnSO4 is added to 15 μL of whole blood and vortex-mixed. Then, 100 μL of a precipitation solution containing 2 μg/L desmethoxyrapamycin (DMR) in acetonitrile is added as an IS. The sample is vortex-mixed and centrifuged, and 20 μL of the supernatant is injected onto a Waters 2795/Quattro Premier XE HPLC-ESI-MS/MS instrument. The analyte is eluted with a gradient of mobile phase A (1 mL/L formic acid and 2 mmol/L ammonium acetate in water) and mobile phase B (1 mL/L formic acid and 2 mmol/L ammonium acetate in methanol), 50% A and 50% B at 0.6 mL/min for 0.4 min, and then 100% B for 0.6 min. The system is then reequilibrated with 50% A and 50% B for 1 min. This procedure produces retention times of 1 min and 1.02 min for SIR and DMR, respectively.

Preliminary postextraction addition experiments in our laboratory indicated that matrix effects cause variable suppression or enhancement of the DMR response in many patient samples. Although postextraction addition experiments and postcolumn infusion studies have been recommended to evaluate and minimize the extent of matrix effects (4), it is unlikely that these effects on ionization can be completely removed in a high-throughput HPLC-ESI-MS/MS method such as the one described above. Recognizing this limitation, we investigated whether the reliability of our assay

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3 Nonstandard abbreviations: HPLC-ESI-MS/MS, HPLC–electrospray ionization–tandem mass spectrometry; IS, internal standard; SIR, sirolimus; DMR, desmethoxyrapamycin; SIR-d3, deuterium-labeled sirolimus; UKNEQAS, United Kingdom National External Quality Assessment Scheme.
could be improved by replacing DMR with deuterium-labeled SIR (SIR-d3) as the IS. A similar strategy has been reported for the measurement of cyclosporin (8).

Our aim was to use both DMR and SIR-d3 as the IS to measure the variation in the response ratio between SIR and each IS for whole-blood samples from actual patients. Previous studies have recommended the use of a variety of individual patient samples to assess IS suitability rather than the use of pooled blood samples, which may fail to account for interpatient matrix variation (6). A secondary aim was to compare the assay results for patient samples obtained with DMR and SIR-d3 as the IS.

To conduct this study, we followed a previously described methodology (4, 5) and used blood from 5 patients who received immunosuppressant drugs other than SIR and had tested negative for SIR (the use of these samples was considered exempt by the internal review committee). The design complies with recommendations by the CLSI (9), the Class II Special Controls Guidance Document for Industry, and the US Food and Drug Administration (10).

In sample set 1, we spiked controls (whole-blood samples from 5 patients not undergoing SIR therapy) with SIR (1.9, 9.5, and 19 μg/L) and the IS (9.4 μg/L DMR or 19.4 μg/L SIR-d3). SIR and DMR reference standard materials were a gift from Wyeth Pharmaceuticals (Pearl River, NY, USA), and SIR-d3 (which may contain up to 5% unlabeled compound) was purchased from Toronto Research Chemicals. DMR and SIR-d3 concentrations were chosen so that the responses for DMR and SIR-d3 produced by the instrument were approximately equivalent. Although unequal responses may arise from different detector responses for each of the compounds in our system, such differences are more likely to be due to difficulties in reconstituting SIR-d3. The manufacturer shipped the compound in glass vials in nominal 1-mg quantities, and we had to dissolve the material in methanol without reweighing. The unlabeled compound present in the labeled material contributed 3% of the SIR-d3 response to the SIR channel. At the SIR-d3 concentrations used in our study, the presence of the unlabeled SIR added approximately 0.3 μg/L to the SIR concentration for all calibrators, controls, and patient samples; therefore, it did not affect the calculated SIR concentrations when either IS was used. Each spiked control was sampled in duplicate and extracted as described above, except that acetonitrile without the IS was used in the precipitation step. In sample set 2, we prepared spiked post-extraction addition samples for the same 5 samples at SIR, DMR, and SIR-d3 concentrations equivalent to those described above by using a fixed volume of the extract obtained after the zinc sulfate/acetonitrile–precipitation step. For sample set 3, we prepared “pure solutions” at SIR, DMR, and SIR-d3 concentrations equivalent to those described above by using a fixed volume of the extract obtained after the zinc sulfate/acetonitrile–precipitation step. Finally, as an additional control we spiked samples of pooled patient blood at an intermediate SIR concentration of 9.5 μg/L.

We assayed replicate, nonsequential injections of each extract from the sample sets by HPLC-ESI-MS/MS and monitored SIR, DMR, and SIR-d3 transitions at m/z 931.6 → m/z 864.6, m/z 901.6 → m/z 816.6,
and m/z 934.6 → m/z 867.6, respectively, to detect all 3 compounds in a single injection.

We used the responses from the 3 sample sets to evaluate matrix effects, absolute recovery, and process efficiency, as described by Matuszewski et al. (4). The data are summarized in Table 1. Although matrix effects and process efficiency varied across compounds and patients, the interpatient CV was consistently lower with SIR-d₃ as the IS than with DMR across the range of SIR concentrations used. The CVs for the 2 IS compounds were similar when the pooled blood sample was used.

The Class II Special Controls Guidance Document for sirolimus test systems also recommends that the possible effects of SIR metabolites on assay performance besides matrix effects should be investigated through the use of blood samples from patients who are taking the drug (11). Thus, we supplemented blood samples from 5 different patients who were undergoing SIR therapy (SIR concentrations of 2.9, 6.5, 10.9, 15.3, and 18.3 μg/L previously measured with DMR as the IS) with additional SIR and assayed the samples as described above. We linearly regressed the response ratio (SIR/SIR-d₃ or SIR/DMR, y axis) for each patient sample against the added SIR concentration (x axis) (SigmaStat, Version 3.5; SPSS). This analysis yielded 2 separate series (one each for SIR-d₃ and DMR) of 5 parallel lines with increasing y intercepts, which correspond to the original SIR concentrations in the samples (data not shown). The CV for the slopes for the SIR-d₃ series was 1.9%, compared with 5.7% for the DMR series. The 95% confidence intervals for the slopes of the individual patients within the SIR-d₃ series overlapped (data not shown), demonstrating the absence of appreciable matrix effects, which in this study include possible effects due to SIR metabolites (4). The data did not support the same conclusion for DMR.

Injector irreproducibility was evaluated by pooling and spiking extracts of whole blood with 3 SIR concentrations and DMR and SIR-d₃ IS concentrations as described above. The pooled spiked extracts were dispensed into 20 individual autosampler vials and injected as outlined above. The CVs for response ratios between SIR and DMR (4.3%, 4.2%, and 2.9%) were lower than the interpatient imprecision at the equivalent SIR concentrations (9.5%, 9.7%, and 7.6%, respectively). This result demonstrates that injector irreproducibility is not solely responsible for the higher interpatient CV with DMR as the IS.

Postextraction solutions of whole blood were spiked with SIR to produce concentrations equivalent to 9–280 μg/L in whole blood. The response was linear with no evidence that high SIR concentrations suppressed the SIR-d₃ signal. To calculate an approximate lower limit of quantification for the assay, we analyzed samples of whole blood spiked with 0.35 μg/L SIR, and

![Fig. 1. Analysis of HPLC-ESI-MS/MS results for SIR in 72 patient samples.](image-url)
the results showed within-run CVs of 10% and 11% for SIR-d$_3$ and DMR, respectively, as the IS.

Finally, we analyzed 72 patient blood samples known to contain SIR (including 9 from the United Kingdom National External Quality Assessment Scheme [UKNEQAS] Sirolimus International Proficiency Testing Scheme) with both DMR and SIR-d$_3$ as the IS. We compared the data with Passing–Bablok regression analysis (Analyze-it Software; Fig. 1A) and a Bland–Altman plot of the numerical difference between the 2 methods for each sample vs the mean of the 2 measurements (12) (Fig. 1B). A previous study used Passing–Bablok plots to compare ISs used to assay everolimus (13), but the use of these plots to distinguish between alternative IS compounds has been questioned (6). Although the Passing–Bablok plot of our data demonstrates a linear relationship between the 2 methods, the mean slope of the regression line (0.82; 95% confidence interval, 0.78 – 0.87) was less than unity, suggesting that the use of DMR overestimates the SIR concentration, compared with the use of SIR-d$_3$. The Bland–Altman plot shows that this difference is consistent across the assay range. Superficially, the data suggest that the use of SIR-d$_3$ as the IS offers more accurate results with less imprecision. This result is consistent with the overestimation of SIR (with DMR as the IS) by our laboratory as a participant in the UKNEQAS Sirolimus International Proficiency Testing Scheme. Accuracy is likely to be influenced by multiple factors, however, including assay methodology, assay calibration, and reference materials. Hence, further investigations are needed to determine whether our apparent overestimation of SIR concentration with the use DMR as the IS, compared with SIR-d$_3$, can be reproduced in other laboratories.

In summary, our investigation has shown that the use of SIR-d$_3$ as the IS provides results with lower interpatient variation compared with the use of DMR. SIR-d$_3$ shows no evidence of metabolite interference and is the preferred IS in this high-throughput HPLC-ESI-MS/MS assay of SIR.

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References


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