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Long-term Cross-validation of Everolimus Therapeutic Drug Monitoring Assays: The Zortracker Study

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Abstract

Background—This ongoing academic collaboration was initiated for providing support to set up, validate, and maintain everolimus therapeutic drug monitoring (TDM) assays and to study long-term inter-laboratory performance.

Methods—This study was based on EDTA whole blood samples collected from transplant patients treated with everolimus in a prospective clinical trial. Samples were handled under controlled conditions during collection, storage, and were shipped on dry ice to minimize freeze-thaw cycles.

For more than 1.5 years participating laboratories received a set of 3 blinded samples on a monthly basis. Among others, these samples included individual patient samples, patient sample pools to assess long-term performance and patient samples pools enriched with isolated everolimus metabolites.

Results—The results between LC-MS/MS and the everolimus Quantitative Microsphere System (QMS, Thermo Fisher) assay were comparable. The monthly inter-laboratory variability (CV%) for cross validation samples ranged from 6.5 – 23.2% (average of 14.8%) for LC-MS/MS and 4.2 – 26.4% (average of 11.1%) for laboratories using the QMS assay. A blinded long-term pool sample was sent to the laboratories for 13 months. The result was 5.31 ± 0.86 ng/mL (range 2.9–7.8 ng/mL) for the LC-MS/MS and 5.20 ± 0.54 ng/mL (range 4.0–6.8 ng/mL) for QMS laboratories.

Conclusions—Enrichment of patient sample pools with 5–25 ng/mL of purified everolimus metabolites (46-hydroxy everolimus and 39-O-desmethyl everolimus) did not affect the results of either LC-MS/MS or QMS assays.

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Conflicts of Interest

None of the other authors declared a conflict of interest.

Both LC-MS/MS and QMS assays gave similar results and showed similar performance, albeit with a trend towards higher inter-laboratory variability among laboratories using LC-MS/MS than the QMS assay.

Key Terms and Phrases

Everolimus; LC-MS/MS; Everolimus Quantitative Microsphere System (QMS) assay; therapeutic drug monitoring; cross validation

INTRODUCTION

Everolimus (RAD001, Novartis, Basel, Switzerland) is an inhibitor of the mammalian target of rapamycin (mTOR) that has a wide spectrum of therapeutic applications, including but not limited to, as immunosuppressant after transplantation, as anti-cancer agent for solid tumors and as proliferation inhibitor coated on drug-eluting stents^{1,2}.

When used as an immunosuppressant after transplantation, everolimus has a narrow therapeutic window and, in most cases, target trough blood concentrations between 3–8 ng/mL have to be maintained^{3,4}. This in combination with an inter-individually variable dose – systemic exposure relationship and intra-individual pharmacokinetic variability mostly due to drug-drug, disease-drug and food-drug interactions requires dose titration and adjustments guided by therapeutic drug monitoring (TDM)⁵.

As for the immunosuppressants cyclosporine, tacrolimus and sirolimus, assays used for clinical TDM of everolimus can be divided in two categories; those based on high-performance liquid chromatography (HPLC) most commonly using a tandem mass spectrometer as detector (LC-MS/MS)⁶ and immunoassays. Although commercial kits are available, most LC-MS/MS TDM assays for everolimus have been developed in-house. The only everolimus immunoassay currently approved in the United States is the Quantitative Microsphere System (QMS) assay (Thermo Scientific/Microgenics, Fremont, CA), a homogeneous particle-enhanced turbidimetric immunoassay⁷. In comparison to the now obsolete Innofluor Certican assay (Seradyn, Indianapolis, IN) the QMS assay is designed to match LC-MS/MS results⁷. The Innofluor Certican assay was a homogeneous fluorescence polarization assay using a polyclonal rabbit antibody⁸ that in comparison to specific LC-MS/MS measurements significantly overestimated everolimus in patient samples⁹ partially due to cross-reactivity with multiple everolimus metabolites¹⁰. The QMS assay better matches LC-MS/MS results by adjusting the calibrators based on a “training” sample set of trough whole blood samples collected from kidney transplant patients⁷. Thus, calibration compensates for cross-reacting metabolites as present in such a training sample set and, accordingly, the assay underestimates spiked blank blood samples as sometimes distributed by proficiency testing programs.

When everolimus was approved by the United States Food and Drug Administration (FDA) in 2010⁵, the challenge with setting up and running Everolimus clinical TDM assays was that patient samples for assay validation were difficult to obtain and that no proficiency testing program meeting United States Clinical Laboratory Improvement Amendments (CLIA, 1988) requirements was available yet. The Zortracker Everolimus TDM Laboratory

Support Program (www.zortracker.com) was designed to address this gap. Zortracker has two main components: a prospective multicenter clinical trial to collect EDTA whole blood samples from transplant patients receiving everolimus immunosuppression and a laboratory component which maintains the sample bank, which assigns LC-MS/MS concentrations to these samples, which makes them available to interested laboratories in the form of “validation kits”, and which provides blinded samples on a monthly basis to participating laboratories for cross-validation.

The goal of the present study was to analyze the performance of participating laboratories during the period of June 2012- February 2014, to compare the performance of the various LC-MS/MS assays with the more standardized QMS assay over time and to assess potential interference caused by unusually high metabolite concentrations as sometimes observed in individual patients.

METHODS

Multi-center clinical trial

The goal of this ongoing open-label, prospective trial is to collect 30 mL EDTA blood samples from kidney and liver transplant patients in all phases after transplantation under standardized conditions to minimize freeze-thaw cycles and to ensure chain of custody to document the location, storage conditions and that the samples have been under control at any given time. The clinical protocol was reviewed and approved by the Colorado Multi-Institutional Review Board (COMIRB, Aurora, CO) as well as the appropriate institutional review boards of the other clinical centers. All subjects have to give their written informed consent. The study is conducted in compliance with all applicable rules of good clinical practices and the Declaration of Helsinki and its amendments. Inclusion criteria are transplant patients who receive everolimus at a clinically appropriate dose; pregnant and nursing female patients and patients with low hematocrits are excluded. Trough EDTA whole blood samples as well as samples 2.0 ± 0.5 hours after everolimus intake (to cover higher concentration ranges) are collected during different patient visits. All samples are stored at -70°C or below within 4 hours after collection and are shipped to the bioanalytical laboratory on dry ice overnight. In addition key demographic and relevant clinical data, including but not limited to, age, gender, type of transplant, time after transplantation, immunosuppressive drug regimen, other co-medications, hematocrit, blood lipids, liver function parameters, rheumatic antibody status, creatinine in serum, diabetes status (Hb1Ac), and known viral infections are documented and managed using the REDCap database¹¹.

Sample processing and generation of validation support and cross-validation samples

The everolimus samples collected in the prospective clinical trial are used to generate samples for two purposes: patient samples that are made available to clinical TDM laboratories in sets of 20 or 40 samples from individual patients for method validation and samples for monthly distribution in the cross-validation study. Samples collected at the University of Colorado Hospital clinical site are picked up after collection and processed immediately to save one freeze-thaw cycle. After receipt from other centers, samples are

immediately stored at -70°C and below until processing. Then samples are thawed (except for samples received from University of Colorado Hospital) and everolimus concentrations are measured using high-performance liquid chromatography—tandem mass spectrometry (LC-MS/MS). The assay and its validation are described in detail in the Supplemental Material. Based on the results and demographic as well as clinical considerations samples may be pooled to generate large-volume samples as required for the long-term pools distributed during the cross-validation study described below. Samples (1 mL) are then aliquoted into 2-mL cryogenic storage vials (Thermo Fisher Scientific, Fair Lawn, NJ) and frozen at -70°C or below again. Validation support and cross-validation samples are shipped to the participating TDM laboratories on dry ice *via* overnight courier. At the time of analysis, samples have undergone two freeze-thaw cycles and, if collected at the University of Colorado Hospital, only one freeze-thaw cycle.

Everolimus metabolites

To assess and compare potential interferences caused by the previously identified major everolimus metabolites¹² with the quantification of everolimus by LC-MS/MS and QMS, these metabolites were generated and isolated as previously described¹⁰. In brief, everolimus was incubated with pooled human liver microsomes, the metabolites were separated and isolated using semi-preparative HPLC/UV, purity of the isolated metabolite fractions was assessed using HPLC-UV-ion trap-MS and the structures of the isolated metabolites were confirmed using ion trap MSⁿ experiments in combination with analysis of the fragmentation patterns as described by Boernsen et al.¹³. In two experiments, cross validation samples were distributed including a pool made from trough EDTA blood samples collected from kidney transplant patients and the same pool spiked with the metabolites. For one experiment, 25 ng/mL of the isolated 39-O-desmethyl-everolimus metabolite and, for a second experiment, 5 ng/mL of 46-hydroxy-everolimus were added.

Cross-validation study

Among individual patient samples, blank samples, spiked samples and the aforementioned pools generated from patient samples to which everolimus metabolites were added, two long-term pool samples were distributed to assess laboratory performance over several months. These long-term pools were distributed for 6 months (June 2012 – November 2012) and 13 months (February 2013 – February 2014). For both pools, trough EDTA whole blood samples from several kidney transplant patients were combined to result in a total volume of 500 mL. Samples were aliquoted and stored at -70°C or below. Each month, three samples were shipped to the participating laboratories, which were blinded. In most of these challenges, one of the samples was from the long-term pools. Between November 2012–February 2014 only patient pools and samples from individual kidney or liver transplant patients and no spiked blank blood samples were distributed to allow for comparison between LC-MS/MS and QMS results in clinical samples. Between 6- 13 TDM laboratories using LC-MS/MS and 4–8 TDM laboratories using the QMS assay reported results each month.

Result reporting, data management and statistical analysis

Results of validation and cross-validation samples are reported *via* the www.zortracker.com website. All test results are entered in ng/mL with one significant figure after the decimal point. In addition, this website also allows for registration of laboratories, for review of validation and cross validation results and for requesting validation sample shipments. Data is deposited into a SQL database and is analyzed using SAS macros (version 9.2, SAS Institute, Cary, NC). The cross validation challenges that consist of three individual blinded samples have been distributed since April 2011 on a monthly basis. Here we report results submitted from laboratories registered with www.zortracker.com, which went online in March 2012. Cross validation reports include the inaccuracy to method mean, inaccuracy-over-time plot and z-value of the individual laboratories results to the mean of all other results using the same method (LC-MS and QMS). Results distribution plots, precision and acceptance (acceptable range ± 3 SD from mean; results with z-value ± 3 were excluded from the statistics) are also provided. In these reports, only results using the same analytical method (LC-MS/MS or QMS) are compared. To analyze and compare LC-MS/MS and QMS performance in the present study, MedCalc (version 14.8.1, MedCalc Software, Ostend, Belgium) and SigmaPlot (version 12.5, SysStat, Richmond, CA) were used. The LC-MS/MS and QMS assay results of patient samples enriched with isolated everolimus metabolites were compared with their corresponding controls using a paired T-test after In-transformation of the data (SPSS, version 22.0, Chicago, IL).

RESULTS

Overall cross validation results

As aforementioned, for 16 months between November 2012 and February 2014, only patient samples (individual or pooled samples) were distributed to facilitate comparison of LC-MS/MS and QMS results. During this period, a total of 735 results were submitted. One hundred data submissions (3 results/submission) were from TDM laboratories using the QMS assay and 145 submissions were from TDM laboratories using LC-MS/MS. Passing-Bablok regression and Bland-Altman plots of the method medians for each sample ($n=48$) showed that LC-MS/MS and QMS results were similar (Figure 1). The regression equation of the Passing-Bablok analysis was $y = 0.073 + 0.961 x$. Bias plots comparing monthly LC-MS/MS and QMS results are shown in Figure 2. Based on outlier statistics, 14 results (3 QMS and 11 LC-MS/MS) were excluded from statistical analysis. In July 2013, a single everolimus-free human whole blood sample was distributed (sample C) and 7/7 QMS and 6/6 LC-MS/MS results were correctly reported as below the lower limit of quantification (LLOQ). As it has been reported that at least for one tacrolimus immunoassay assay the difference between immunoassay and LC-MS/MS is greater close to the LLOQ with the immunoassay overestimating tacrolimus concentrations¹⁴, a total of 7 patient samples with everolimus concentrations below the lower limit of the therapeutic range (3 ng/mL)^{3,4} were distributed. There was no statistically significant difference between LC-MS/MS and QMS results (medians, LC-MS/MS ($n=8-12$)/QMS ($n=5-8$): 1.8/1.7, 2.4/2.5, 2.4/2.7, 2.2/2.2, 2.8/3.0, 2.7/2.9 and 2.5/2.7 ng/mL). For these samples, 3 of 44 QMS results (6.8%) were reported as below LLOQ and 3 of 69 LC-MS/MS results (4.3%). There was also no significant difference in precision in these low samples between LC-MS/MS and QMS

assays. The average coefficient of variation was 17.1% (range 15.2 – 18.3%) for LC-MS/MS and 15.8% (range 8.8 – 25.3%) for QMS.

Long-term pools

A total of 70 LC-MS results and 40 QMS results for long-term pool 1 (distributed June 2012– November 2012) and 107 LC-MS results and 77 QMS results for long-term pool 2 (distributed February 2013–February 2014) were reported. The concentrations reported by TDM laboratories using LC-MS/MS were mean $\pm$ standard deviation 5.08 ± 0.77 ng/mL (range 3.5–7.3 ng/mL, 10–13 laboratories returned results each month) and 5.13 ± 0.54 ng/mL (range 4.0–6.2 ng/mL) for the TDM laboratories using the QMS assay (4–8 laboratories each month). For long-term pool 2, which was distributed for 13 months, the results reported by TDM laboratories using LC-MS/MS was 5.31 ± 0.86 ng/mL (range 2.9–7.8 ng/mL with 6–10 laboratories reporting each month) and 5.20 ± 0.54 ng/mL (range 4.0–6.8 ng/mL with 4–8 laboratories reporting each month) for the TDM laboratories using the QMS assay. Frequency distributions of the LC-MS/MS and QMS results for both long-term pools are shown in Figure 3. Figure 4 shows a comparison of the LC-MS/MS and QMS results as reported each month.

Everolimus metabolites

To test for potential interference with LC-MS/MS and QMS assays by everolimus metabolites, a pool of blood samples from kidney transplant patients was spiked with 39-O-desmethyl everolimus or 46-hydroxy everolimus and results were compared to samples from the same pool without addition of the metabolites (control). Controls and metabolite-enriched samples were distributed in the same cross-validation challenge. The metabolites at the concentrations tested did neither significantly affect the results of the LC-MS/MS nor of the QMS assays (Figure 5).

DISCUSSION

The present study was based on EDTA whole blood samples collected from everolimus-treated kidney and liver transplant patients in a prospective multi-center clinical trial. Samples had undergone two freeze-thaw cycles or less. This distinguished our study from other cross-validation and proficiency testing programs that often use left-over samples, artificial matrices, and spiked blank blood and that often lack a chain of control and do not control freeze-thaw cycles, storage, and shipping conditions of the circulated test samples. In the present study, the use of samples that were as close as possible to blood samples typically used for TDM allowed for assessing clinical TDM laboratory performance over 14 months and comparison of results in clinical samples as reported by laboratories using LC-MS/MS and QMS assays over this time period.

Due to chromatographic separation and the specificity of the detector, LC-MS/MS allows for specific quantification of everolimus and other immunosuppressants. In contrast, the antibodies used for clinical TDM immunoassays cross-react with the immunosuppressant metabolites in blood to various extent resulting in concentrations higher than those measured using LC-MS/MS (positive bias). The QMS assay is no exception⁷. However, although the

antibody of the QMS assay is known to cross-react with several everolimus metabolites⁷, in the present study QMS and LC-MS/MS assays consistently gave similar results. This can be explained by the fact that, as aforementioned, the QMS calibrators are adjusted based on the LC-MS/MS results of a training set of trough samples from adult renal transplant patients⁷, thus compensating for the average positive bias caused by the metabolites. Nevertheless, a previous study had observed a positive bias of 11% of the QMS assay compared to LC-MS/MS¹⁵. Such a positive bias was confirmed by two more recent studies^{16,17}. However, these were single laboratory studies and sample quality and storage conditions were not as rigorously controlled as in the present study. While the present study was mostly based on samples from kidney transplant patients, these studies^{16,17} included a relatively large number of samples from liver and lung transplant patients.

At least in the case of tacrolimus and cyclosporine, liver transplant patients tend to have higher metabolite concentrations in blood than stable kidney transplant patients^{18,19} and it is reasonable to expect that this is also the case with everolimus. It has to be noted that the value-assigned QMS calibrators are based on a training set from kidney transplant patients. A positive bias of up to 10% in liver transplant patients was also reported by the manufacturer of the QMS assay⁷. Due to the small number of samples from liver transplant patients distributed (Figure 2), in the present study statistical comparison of the performances of LC-MS/MS and QMS assays in these samples was not feasible.

A trend towards higher variability among laboratories using LC-MS/MS than laboratories using QMS everolimus assays was observed. For the results reported during the time period November 2012 and February 2014 (Figure 2), an average coefficient of variation (CV%) of 14.8% (range 6.5 – 23.2%) for LC-MS and 11.1% (4.2 – 26.4%) for QMS assays was observed. This is in the same range as observed for LC-MS/MS and immunoassays for other immunosuppressants such as tacrolimus²⁰, including a higher inter-laboratory variability among TDM laboratories using LC-MS/MS in comparison to those using immunoassays^{20,21}. It has been discussed that this may be due to the fact that immunoassays are highly automated and standardized, while LC-MS/MS assays are in most cases laboratory-developed and often use calibrators that are prepared in-house. However, standardization of the calibrators has not always remedied the accuracy and inter-laboratory variability of LC-MS/MS assays^{21,22}. There is evidence that in addition differences in sample preparation and vulnerability of electrospray ionization to matrix effects may play a role²².

One of the frequently mentioned disadvantages of immunoassays used for the TDM of immunosuppressants is the cross-reactivity of their antibodies with metabolites, resulting in a significant positive bias. To assess the effect of two metabolites, 46-hydroxy everolimus and 39-O-desmethyl everolimus, were added to samples from a pool of kidney transplant patient blood samples. 46-hydroxy everolimus was selected as it is the major everolimus metabolite in blood of kidney transplant patients with a reported median of 44.1% of the measured everolimus trough blood concentration¹². 39-O-desmethyl everolimus was selected for several reasons. Its cross-reactivity is not included in the product information of the QMS assay⁷ and with 43% it was among the metabolites with the highest cross-reactivities with the Innofluor everolimus immunoassay¹⁰, and it has an HPLC retention

time very close to that of everolimus, so that it may be possible that it affects everolimus quantification by LC-MS/MS assays that do not chromatographically separate 39-O-desmethyl everolimus and everolimus, e.g. by interfering during the electrospray ionization process. Interference by these metabolites with neither LC-MS/MS nor the QMS assay were observed. In the case of 46-hydroxy everolimus and the QMS assay this can be explained by the low cross-reactivity of this metabolite of 0–2%⁷. Our data also suggests that the QMS assay does not cross-react with 39-O-desmethyl everolimus.

It is concluded that in the present study LC-MS/MS and QMS everolimus TDM assays gave similar results and showed similar performance, albeit with a trend towards higher inter-laboratory variability among those laboratories using LC-MS/MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Passing-Bablok regression (top) and Bland-Altman plot (bottom) of the QMS and LC-MS/MS results in clinical samples from everolimus-treated transplant patients circulated between November 2012 and February 2014

Three samples were sent to the participating TDM laboratories every month for 16 months. The median results for each sample (n=48) are plotted. One of the distributed samples was a blank blood sample spiked with 16 ng/mL everolimus (marked by arrow in both plots). As the calibrators of the QMS are value-assigned based on patient samples that also contain everolimus metabolites, the QMS assay measured this sample lower than the LC-MS/MS assays.

Figure 2. Comparison of laboratory cross-validation results from TDM laboratories using LC-MS/MS and QMS everolimus assays

All samples were pools or individual EDTA whole blood samples from kidney and liver transplant patients (marked by arrows) that were collected under controlled conditions in the prospective clinical trial and had undergone two or less freeze-thaw cycles. The box and whisker plots show the % bias in reference to the mean of the pooled LC-MS/MS and QMS results after exclusion of outliers. This was justified as the QMS calibrators were adjusted by the manufacturer based on LC-MS/MS results to ensure that QMS and LC-MS/MS results match⁷. The limits of the box are the 25 and 75% percentiles, the error bars represent the 10% and 90% percentiles. Medians and mean lines (thicker line) are shown. Individual data points below the 10% percentile and above the 90% percentile are shown as “x”.

Long-term pool distributed June 2012 – November 2012

Long-term pool distributed February 2013 – February 2014

Figure 3. Comparison of the frequencies of everolimus concentrations reported for the two long-term pool samples distributed during the cross-validation study as measured by LC-MS/MS and QMS assays

The two long-term pool samples were distributed monthly for 6 and 13 months.

Figure 4. Comparison of the monthly cross-validation results for the two long-term pool samples as reported by TDM laboratories using LC-MS/MS and QMS everolimus assays

The box and whisker plots show the % bias in reference to the mean of the pooled LC-MS/MS and QMS results after exclusion of outliers. Samples from pool 1 were distributed monthly between June 2012 and November 2012 and samples from pool 2 between February 2013 and February 2014.

Figure 5. Lack of interference of the metabolites 39-O-desmethyl everolimus and 46-hydroxy everolimus with the quantification of everolimus using LC-MS/MS and QMS assays
 25 ng/mL isolated 39-O-desmethyl everolimus (top) or 5 ng/mL isolated 46-hydroxy everolimus (bottom) were added to a pool of blood samples from kidney transplant patients. The results were compared with those from samples of the same pool without isolated metabolites added (control). Control and metabolite samples were distributed together in the same monthly cross-validation challenge. Results are presented as % bias in reference to the everolimus concentrations mean of the pooled LC-MS/MS and QMS results of the control samples (box and whisker plots). No significant increase in bias for the metabolite versus the

corresponding control samples was observed, suggesting a lack of interference by the metabolites with everolimus quantification at the concentrations tested.

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