Standardization of LC-MS for Therapeutic Drug Monitoring of Tacrolimus

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BACKGROUND: LC-MS is increasingly used for therapeutic drug monitoring of tacrolimus. A recent summary from an international proficiency-testing scheme demonstrated that the mass spectrometry respondents were the largest method group. However, these methods lack standardization, which may explain the relatively poor interlaboratory agreement for such methods. This study aimed to provide one path toward the standardization of tacrolimus quantification by use of LC-MS.

METHODS: A 40-member whole blood tacrolimus proficiency panel was circulated to 7 laboratories, 4 in the US and 3 in Europe, offering routine LC-MS–based quantification of tacrolimus. All laboratories used a common LC-MS platform and followed the manufacturer’s instructions that accompanied an LC-MS reagent kit intended for tacrolimus quantification in whole blood samples. Four patient pools were prepared that had sufficient volume to allow comparison with a tacrolimus reference measurement procedure.

RESULTS: For the 40-member panel, the standardized MassTrak LC-MS assay demonstrated excellent agreement with a validated LC-MS method used by Analytical Services International (y = 1.02x – 0.02; r = 0.99). The CVs for the pooled patient samples ranged from 2.0% to 5.4%. The mean difference from the reference measurement procedure ranged from 0.4% to 4.4%.

CONCLUSIONS: Tacrolimus assay standardization, which must include all facets of the analysis, is necessary to compare patient results between laboratories and to interpret consensus guidelines. LC-MS can provide accurate and precise measurement of tacrolimus between laboratories.

 lc-MS has enjoyed robust growth in clinical laboratories for the routine measurement of a wide variety of analytes. Improved analytical specificity compared to immunoassays, and the ability to multiplex assays, have hastened the adoption of this technique. However, at present most LC-MS assays are laboratory-developed tests that require in-house method development, calibration, and validation. To ease the adoption of LC-MS technology into clinical practice, fragmented resource materials must be compiled and clarified, and generalized validation procedures must be adapted for specific application to LC-MS. This has led to assays that, although well suited for use within individual laboratories, may lack agreement between laboratories—confounding clinical research and hindering the adoption of common clinical practice guidelines.

The immunosuppressant tacrolimus is a calcineurin inhibitor that is widely used for the prophylaxis of allograft rejection in solid organ transplant recipients. Monitoring trough whole blood concentrations, the preferred specimen in clinical settings (1), is generally regarded as a good surrogate for tacrolimus exposure. Although dose adjustments critical to regulating the degree of immunosuppression are made, in part on the basis of laboratory results, precise therapeutic ranges have yet to be established (2). It is possible that the concentration–effect relationship for tacrolimus has not been better defined because of the variety of analytical methods for measuring tacrolimus in use today and the lack of their standardization and traceability to a single defined source.

Based on the catchment area of one international proficiency testing service (3), LC-MS is now the most widely used method for tacrolimus measurement. However, as demonstrated in a recent study comparing tacrolimus quantification by several methods (4), laboratory-developed LC-MS assays lack standardization in terms of methodology (e.g., calibration,

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extraction, analytical column, chromatography, instrumentation), leading to large between-laboratory imprecision and inaccuracy. Therefore, in this study we used a commercially available LC-MS assay kit for tacrolimus, the Waters® MassTrak™ immunosuppressants kit, a common LC-MS instrument platform, and a proficiency-testing survey of laboratories using this assay to assess whether interlaboratory imprecision could be improved through standardization of LC-MS analysis. Further, we compared the results obtained using this test system to a reference measurement procedure for tacrolimus and estimated the method’s measurement uncertainty by repeat measurement of a higher-order reference material.

Materials and Methods

TACROLIMUS PROFICIENCY-TESTING PANEL
A whole blood tacrolimus panel was prepared by Analytical Services International (ASI) as previously described. Briefly, 10 patient pools (target 0–25 ng/mL) and 10 tacrolimus-supplemented samples (target 2–25 ng/mL) were prepared in duplicate. The tacrolimus-supplemented samples were prepared in EDTA anticoagulated human whole blood from Biological Specialty. Tacrolimus hydrate (99.7% purity) was purchased from Enzo Life Sciences. Pooled patient samples were from kidney transplant patients receiving tacrolimus. The samples were pooled from the volumes remaining after routine therapeutic drug monitoring and would otherwise have been discarded. The resulting 40 samples were randomized and distributed blindly to the participating centers. Four of the patient pools were prepared in sufficient volume (approximately 20 mL) to allow for value assignment by an exact-matching isotope dilution mass spectrometry (EM-IDMS) method (the reference measurement procedure) at LGC.

PARTICIPATING CENTERS AND INSTRUMENTATION
The sole inclusion criterion for this study was the availability of a common LC-MS platform, namely the ACQUITY® TQD UPLC®-tandem quadrupole MS system (Waters Corporation). Participating centers were provided with the MassTrak immunosuppressants kit. This reagent kit is indicated for the quantification of tacrolimus in liver and kidney transplant patient whole blood samples and is CE marked in Europe and cleared by the US Food and Drug Administration. The kit contains 6 primary assay calibrators (5 nonzero and 1 blank whole blood calibrator), 3 levels of QC material, an internal standard (ascomycin), an instrument-tuning solution, a chromatography column, and directions for use. Thus, assay calibration, sample pretreatment, and instrumentation were standardized among the participating centers. This level of platform standardization was consistent with the Abbott Architect and Siemens/Dade Dimension tacrolimus assays that were the subject of an earlier study, but differed from the variety of tacrolimus LC-MS measurement procedures used in that same study. The MassTrak assay performance characteristics have been described elsewhere.

SAMPLE ANALYSIS
Samples were analyzed initially at ASI by an LC-MS method used for value assignment of samples distributed by the International Proficiency Testing Scheme for tacrolimus. This method has previously been shown to be in good agreement with the EM-IDMS method used at LGC.

Participating laboratories analyzed the samples in the test panel as unknowns, following the directions for use that accompany the test kits, and as previously described.

DATA ANALYSIS
Data from the participating laboratories were used to estimate interlaboratory imprecision. The data were also compared to the ASI method as an initial measure of accuracy. Accuracy was further evaluated for a subset of the patient pools by comparing the mean concentrations from the 7 laboratories to the EM-IDMS method.

ANALYSIS OF TACROLIMUS CERTIFIED REFERENCE MATERIAL
To further assess the accuracy of the MassTrak assay, and to derive a preliminary estimate of measurement uncertainty, 6 vials of a certified tacrolimus reference material in human whole blood [European Reference Material (ERM)-DA110a, LGC Standards] were analyzed by the tacrolimus assay manufacturer at their Manchester, UK, facility. ERM-DA110a is listed on the Joint Committee for Traceability in Laboratory Medicine database for higher-order reference materials. The 6 vials were analyzed in triplicate on 3 separate days using the MassTrak assay system described above.

STATISTICS
Passing–Bablok regression analysis, Pearson correlation analysis, and Bland–Altman bias estimation were calculated using Analyse-it for Microsoft Excel (version 2.30).
Results

TACROLIMUS PROFICIENCY TESTING PANEL

Table 1 lists the mean, %CV, and range of results returned by the participating centers. For comparison, we also included in this table the summary data reported by Levine et al. (4) for the Abbott Architect analyzer, which was the best-performing immunoassay in their study. For tacrolimus-supplemented samples the range of imprecision (%CV) was 3.7% to 12.2% (Table 1). One whole blood sample was a blank sample not supplemented with tacrolimus, and no center reported any tacrolimus in that sample above the lower limit of quantification of the assay (0.38 ng/mL). The lower limit of quantification was defined as the lowest concentration at which both the CV and bias from anticipated values of QC material were <20% (5).

For patient pools, which more closely represent true patient samples vs supplemented samples, the %CV for the standardized MassTrak LC-MS assay ranged from 2.0% to 5.4%, with a mean of 4.3% (Table 1). For all of the supplemented samples and patient pools, the mean interlaboratory CV for the standardized LC-MS assay was 5.5% compared to a mean CV of 6.4% for the Architect assay in the earlier, comparable study. In the interest of disclosure, we did not include the data for one supplemented sample from the study by Levine et al. (30 ng/mL, mean Architect interlaboratory CV 8.6%) (4), nor did we include a supplemented sample from our study (12 ng/mL; mean LC-MS interlaboratory CV, 5.0%). Had we included these 2 samples, the overall mean CV for the Architect would have increased slightly and the mean CV for the LC-MS assay would have decreased slightly. However, because we did not have a direct comparison for these 2 samples, we removed these samples from our analysis of the data.

Table 2 lists the mean values for patient pools P-02, P-04, P-06, and P-09 obtained by the MassTrak assay, the value reported by the ASI method, and the value assigned by the EM-IDMS method.
age difference from the ASI method ranged from 0% to 2.6%, and the percentage difference from the EM-IDMS ranged from 0.4% to 4.4%, demonstrating excellent agreement with both measurement procedures across the generally accepted therapeutic range for tacrolimus. These data are also presented graphically in Fig. 1 as box-and-whisker plots with the EM-IDMS measurement and associated uncertainty overlaid.

**Table 2. Comparisons of the MassTrak LC-MS method to ASI LC-MS and EM-IDMS methods.**

<table>
<thead>
<tr>
<th>Panel</th>
<th>MassTrak LC-MS&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>ASI LC-MS&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Difference, %&lt;sup&gt;c&lt;/sup&gt;</th>
<th>EM-IDMS&lt;sup&gt;b,d&lt;/sup&gt;</th>
<th>Difference, %&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-02</td>
<td>4.6 (0.21)</td>
<td>4.5</td>
<td>2.2</td>
<td>4.58 ± 0.12</td>
<td>0.4</td>
</tr>
<tr>
<td>P-04</td>
<td>8.0 (0.32)</td>
<td>7.8</td>
<td>2.6</td>
<td>7.66 ± 0.19</td>
<td>4.2</td>
</tr>
<tr>
<td>P-06</td>
<td>12.4 (0.59)</td>
<td>12.1</td>
<td>2.5</td>
<td>11.90 ± 0.31</td>
<td>4.4</td>
</tr>
<tr>
<td>P-09</td>
<td>20.7 (1.03)</td>
<td>20.7</td>
<td>0.0</td>
<td>19.82 ± 0.40</td>
<td>4.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Measured values are expressed as the mean (SD) of the mean at the 95% CI (n = 7 sites).
<sup>b</sup>Concentrations are expressed in nanograms per milliliter.
<sup>c</sup>Between MassTrak (MT) LC-MS and ASI LC-MS method: (MT - ASI)/ASI × 100.
<sup>d</sup>EM-IDMS values are expressed as the reference value ± expanded uncertainty of the measurement. Expanded uncertainty includes all aspects of the measurement procedure, including the uncertainty associated with the purity of the standard material used, enabling traceability to the SI and is expressed at the 95% CI.
<sup>e</sup>Between MassTrak (MT) LC-MS and EM-IDMS method: (MT - EM-IDMS)/EM-IDMS × 100.

**Fig. 1. Box-and-whisker plots for patient pools P-02, P-04, P-06 and P-09.**

The left boundary of the box represents the first quartile of results, the right boundary represents the third quartile of results, and the line intersecting the box represents the median value of results from the 7 laboratories. The whiskers represent the lowest and highest values returned by any laboratory. For comparison, the reference values (solid line) and associated measurement uncertainties (dashed lines) for the EM-IDMS method are overlaid.
Fig. 2 shows the combined method comparison data (MassTrak LC-MS vs the ASI LC-MS assay) for the 7 laboratories. For the 40-member panel set the MassTrak assay demonstrated overall excellent agreement (y = 1.02x − 0.02; r = 0.99) with the validated LC-MS method used by ASI. The method comparison data (MassTrak LC-MS vs the ASI LC-MS assay) for each of the 7 laboratories are shown in Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue11.

ANALYSIS OF TACROLIMUS CERTIFIED REFERENCE MATERIAL
The concentration of the tacrolimus reference material (ERM-DA110a), as certified by its manufacturer (LGC), was 7.82 (0.25) ng/mL (expanded uncertainty, 95% CI). The mean tacrolimus concentration as measured by the MassTrak assay at the manufacturer’s site was 8.1 (0.43) ng/mL [mean (SD); n = 54]. The percentage bias between the mean MassTrak data and the certified value of the certified reference material (CRM) was determined to be 3.6%. The percentage bias expanded measurement uncertainty, using the “top down” approach described by Barwick (8), was calculated to be 3.8%, greater than the percentage bias between the certified value and manufacturer’s measured value, and therefore the bias was considered to be insignificant. A method measurement uncertainty was calculated by combining the bias uncertainty with the SD of all data generated to date, using the root sum of the squares, resulting in an expanded uncertainty of 11% (at the 95% CI).

Fig. 3 shows the excellent agreement of the MassTrak assay with the certified value of the CRM. When the expanded measurement uncertainty of 11% was applied to each of the individual MassTrak measurements (error bars) the certified value of the CRM was encompassed in all cases.

Discussion
Improved analytical specificity compared to immunoassays (9, 10) and the ability to multiplex assays (11, 12) have hastened the adoption of LC-MS into clinical laboratories. Most LC-MS assays are laboratory-developed tests that include in-house method development, calibration, and validation. These assays lack standardization, especially in calibrator value assignment, which contributes to greater interlaboratory imprecision. Therefore, in this study we used a commercially available LC-MS assay kit for tacrolimus, a common LC-MS instrument platform, and a proficiency testing survey of laboratories using this assay, to assess whether interlaboratory imprecision could be improved through standardization of LC-MS analysis. Further, we compared the results obtained us-
ing this test system to a reference measurement procedure for tacrolimus.

The results of our study demonstrate that the standardization of key analytical variables (calibration materials, sample pretreatment protocols, and chromatography) in the LC-MS analysis of tacrolimus yields highly reproducible tacrolimus measurements across laboratories. This standardization was achieved through the use of the MassTrak immunosuppressants assay kit on a common LC-MS instrument platform. In addition, the MassTrak immunosuppressant assay for tacrolimus demonstrated excellent agreement with both a higher-order measurement procedure (ASI) and a reference measurement procedure having a very small measurement uncertainty (LGC). The technique has also been shown to be free from the many factors that negatively impact commercially available immunoassays, namely tacrolimus metabolites (13), hematocrit and serum albumin (14–16), and heterophilic antibodies (17). Although LC-MS has traditionally been considered a cumbersome technique, one relegated to the research laboratory, the rapid sample pretreatment and chromatography afforded by modern LC-MS instrumentation have led Taylor et al. to conclude recently that LC-MS for tacrolimus therapeutic drug monitoring truly is a routine test (18).

A limitation of the comparison of the standardized LC-MS assay results in this study with those of the best-performing immunoassay from published data, the Abbott Architect assay (4), is that whereas the nominal concentrations of tacrolimus for the supplemented samples and the target concentrations of tacrolimus in the patient pools were matched, the samples in our study were not the exact same samples used to evaluate the Architect assay. Nonetheless, we believe that the excellent interlaboratory imprecision and accuracy of results with the standardized MassTrak LC-MS assay are self-evidently clear from our data.

The concept of measurement uncertainty is gradually being understood, appreciated, and adopted by clinical laboratories, in part owing to one of the requirements of the ISO 15189 standard (Medical Laboratories—Particular Requirements for Quality and Competence), which is that measurement uncertainty for quantitative assays should be calculated (19). With the use of the data generated from the analysis of the CRM over 3 separate days, a preliminary calculation of the MassTrak assay’s measurement uncertainty at the
Manchester laboratory was made. It should be noted that this is just a preliminary evaluation of measurement uncertainty, and a more accurate estimate can be determined once a study has been performed over an extended period of time and factors such as analyst-to-analyst variability and new batches of calibration materials are encompassed.

The need for assay standardization in LC-MS analyses goes beyond the quantification of tacrolimus. Another widely monitored analyte is serum 25-hydroxyvitamin D. Although LC-MS quantification of 25-hydroxyvitamin D has been shown to be superior to immunoassay in accuracy (20, 21), calibration differences have been shown to affect interlaboratory agreement of results. The use of a common calibrator in 25-hydroxyvitamin D analyses markedly reduces interlaboratory imprecision (22, 23). However, the use of a common calibrator alone may not maximize interlaboratory agreement, as demonstrated for tacrolimus by Levine et al. (4). LC-MS analysis involves other variables such as the sample and reagent volumes, sample preparation protocols, chromatography, and mass transitions monitored; these variables were not standardized in the earlier study on tacrolimus measurement.

New discoveries in the pathophysiology, diagnosis, and treatment of diseases are emerging from omics studies. To make the transition from research to clinical application, acceptable accuracy, reproducibility, and interlaboratory transferability will need to be achieved. In this regard, the use of standardization kits has helped identify procedural errors in plasma proteomic analyses by multiple-reaction monitoring MS (24). These reference kits, when used according to recommended settings and standard operating protocols, have been shown to allow the reliable and precise MS quantification of 40 peptides.

In summary, although current LC-MS assays may lack optimal interlaboratory accuracy and imprecision, our study demonstrates that improved interlaboratory accuracy and imprecision can be achieved through standardization of the LC-MS analysis. This level of standardization represents a major improvement over both immunoassays and laboratory-developed LC-MS tests for tacrolimus therapeutic drug monitoring and should ease the translation of results from clinical trials and consensus guidelines into routine clinical practice. Furthermore, an accurate and precise technique for monitoring tacrolimus may help to better define the concentration-effect relationship for this drug that has been ill-defined to date (2). We believe this to be the first study to demonstrate standardization of an LC-MS assay for tacrolimus across multiple laboratories, and it could provide a model for future studies that aim to quantify the impact of individual parameters that contribute to the variability in LC-MS assays. Of course, standardization is not a substitute for good laboratory practices such as internal assay validation and system performance checks, which are also key elements for generating accurate and precise results.

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References


