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Multicenter Evaluation of a New Electrochemiluminescence Immunoassay for Everolimus Concentrations in Whole Blood

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Background: The precise monitoring of everolimus, an immunosuppressant drug, is vital for transplant recipients due to its narrow therapeutic range. This study evaluated the analytical performance of a new electrochemiluminescence immunoassay (ECLIA) for everolimus concentrations in whole blood.

Methods: Accuracy, imprecision, and sensitivity studies for the Roche Elecsys everolimus ECLIA were performed at 5 European laboratories. The ECLIA was compared with liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods, as well as the Quantitative Microsphere System everolimus assay.

Results: Everolimus ECLIA accuracies were within the range 100% ± 9%. Coefficients of variation (CVs) across the target range were ≤4.8% for repeatability and ≤8.4% for intermediate imprecision, whereas multisite reproducibility at lower (2.71 mcg/L) and higher everolimus concentrations (3.0–30.0 mcg/L) resulted in CVs of ≤13.7% and ≤12.4%, respectively. The CV at the assay's lower limit of quantification without considering bias was excellent, estimated as ≤9.3% at 0.5 mcg/L. The weighted Deming regression analysis, used for comparison of the results obtained by everolimus ECLIA and by LC-MS/MS methods, yielded a slope of 1.21 [95% confidence interval (CI): 1.15–1.26], intercept of 0.478 mcg/L (95% CI: 0.241–0.716), and a Pearson correlation coefficient (r) of 0.91. A single-site comparison between the ECLIA and the Quantitative Microsphere System assay revealed a slope of 1.05 (95% CI: 0.917–1.17), intercept of 1.03 mcg/L (95% CI: 0.351–1.70), and r of 0.91.

Conclusions: Based on these results, the Roche Elecsys everolimus ECLIA can be considered suitable for routine therapeutic drug monitoring. A positive bias was observed with respect to LC-MS/MS methods, suggesting that it may be necessary to rebaseline individual patients when switching from LC-MS/MS to the ECLIA; however, this must also be considered for any change of method for everolimus measurement.

Key Words: everolimus, immunoassay, LC-MS/MS, therapeutic drug monitoring, ECLIA

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INTRODUCTION

Everolimus is a synthetic derivative of the mammalian target of rapamycin inhibitor, sirolimus, with a superior pharmacokinetic profile.[1] It is indicated for all solid transplant recipients.[2,3] Therapeutic drug monitoring (TDM) of everolimus is essential due to its narrow therapeutic index, interindividual and intraindividual variations in bioavailability and clearance, and potential for interaction with concomitantly administered drugs. Furthermore, the need to take everolimus continuously to prevent organ rejection necessitates to obtain precise and accurate results of the concentration in a patient's blood.[4]

Given the good correlation between exposure to everolimus (area under the concentration–time curve) and trough concentration, measuring trough concentrations is considered an appropriate strategy for TDM of everolimus in the transplant setting. Recently published guidelines have suggested that, in general, trough concentrations of everolimus should be in the therapeutic range of 3–8 mcg/L when used in combination with other immunosuppressive drugs such as

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calcineurin inhibitors and glucocorticoids, whereas in calcineurin inhibitor–free regimens, the therapeutic range should be 6–10 mcg/L.[5,6]

Fully validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods, with a lower limit of quantification (LLoQ) close to 1.0 mcg/L, are considered the methods of choice for monitoring trough concentrations of everolimus in whole blood.[5] However, immunoassays offer an alternative approach to LC-MS/MS methods with potential for less complexity, automation, and round-the-clock results.[7] To the best of our knowledge, limited published information is available on the use of these immunoassays in routine clinical practice.

In this multicenter study, we studied different analytical performance characteristics (inaccuracy, imprecision, and LLoQ without considering bias) to evaluate the Elecsys Everolimus assay, a new electrochemiluminescence immunoassay (ECLIA). In addition, a study was performed to test interchangeability between results obtained by the everolimus ECLIA and by LC-MS/MS methods, as well as by a QMS assay.

MATERIALS AND METHODS

Investigational Sites and Instruments

This study was initiated between January and May 2015 at 5 centers in Europe; 2 in Germany (Central Institute for Laboratory Medicine and Clinical Chemistry, Klinikum-Stuttgart, Stuttgart, and Hospital of the University of Munich, Munich), 2 in Belgium (Ghent University Hospital, Ghent, and Cliniques universitaires St. Luc, Brussels), and 1 in Spain (Hospital Universitari de Bellvitge, Barcelona). All centers were experienced in the TDM of immunosuppressive drugs. The ECLIA evaluation was performed using *cobas e* 411 instruments at Stuttgart, Munich, Brussels, and Ghent. The *cobas e* 601 system was used in Barcelona. All 5 sites used LC-MS/MS method for routine measurement of everolimus concentrations. The Ghent site also used an Indiko Plus analyzer (Thermo Scientific, Waltham, MA) and the QMS everolimus assay (Thermo Scientific) for everolimus method comparison experiments.

Ethical Approval

Independent ethics committee approval was obtained before study initiation at each of the 5 centers (Central Institute for Laboratory Medicine and Clinical Chemistry, Klinikum-Stuttgart; Hospital of the University of Munich; Ghent University Hospital; Cliniques universitaires St. Luc; and Hospital Universitari de Bellvitge) and the study was conducted according to the Declaration of Helsinki (as amended in Tokyo, Venice, and Hong Kong) and International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use Good Clinical Practice guidelines.

Samples

For the inaccuracy experiments, commercial proficiency testing samples from the International Proficiency Testing (IPT) Immunosuppressant Scheme (LGC Standards, Bury, Lancashire, United Kingdom) were used.[8]

PreciControl Everolimus (PC E 1–3; Roche R&D, Penzberg, Germany) from Roche Diagnostics (Roche Diagnostics GmbH, Rotkreuz, Switzerland) at concentrations of 2.71 mcg/L (PC E L1), 9.84 mcg/L (PC E L2), and 20.1 mcg/L (PC E L3) were used to estimate imprecision. Also, 5 human sample pools (HSP; whole blood) within the concentration range of 0.5–30 mcg/L (HSP2–6) were used.

To estimate the LLoQ without considering bias of the ECLIA, the following 11 spiked HSP samples (whole blood) that covered an everolimus target concentration range from 0.2 to 2.0 mcg/L were used: HSP7, 2.0 mcg/L; HSP8, 1.8 mcg/L; HSP9, 1.6 mcg/L; HSP10, 1.4 mcg/L; HSP11, 1.2 mcg/L; HSP12, 1.0 mcg/L; HSP14, 0.8 mcg/L; HSP15, 0.4 mcg/L; HSP16, 0.3 mcg/L; and HSP17, 0.2 mcg/L.

Anonymized, residual whole blood samples, originally collected by a single venipuncture into plasma tubes with ethylenediaminetetraacetic acid-K_3, from patients (native samples) under everolimus therapy who had received either a kidney, liver, or heart transplant, were used for the interchangeability studies (method comparison experiments). In addition, leftover anonymized samples from the Medizinische Hochschule Hannover were used to reach the targeted sample numbers for each transplant type in Ghent and Munich. Samples, if tested within 5 days of collection, were allowed to be stored at room temperature (18–25°C) or at 2–8°C for up to 1 week. Where longer storage was necessary, samples were frozen at between −15 and −25°C, or ideally at −80°C, if available. Whole blood aliquots for method comparisons were used within 24 hours and then stored at 2–8°C between measurements.

Procedures of Assays

The Elecsys Everolimus ECLIA (Roche Diagnostics), which uses the principle of electrochemiluminescence for measurement and is for use on the *cobas e* analyzers (Roche Diagnostics), was performed according to instructions from the manufacturer.[9] Briefly, the material to be measured (calibration, internal quality control [QC], and whole blood samples) are equilibrated to room temperature (18–25°C) and mixed, without vortexing, immediately before use. The material to be measured (300 μL) is combined with 300 μL of Elecsys ISD Sample Pretreatment Reagent (Roche Diagnostics) in a microcentrifuge tube, capped, and vortexed for at least 10 seconds. The samples are then centrifuged for 4 minutes at ≥10,000*g*. The supernatant is decanted into an appropriate vial and capped until loading onto the system. Only 35 μL of supernatant is aspirated by the analyzer. The total assay duration is 18 minutes.

The Elecsys Everolimus ECLIA is calibrated using the Roche Elecsys Everolimus CalSet (Roche Diagnostics) with 2 concentrations (0.6 and 23.7 mcg/L). Calibrators were reconstituted according to the manufacturer's instructions[10] and stored in 300 μL aliquots at 2–8°C or below –15°C. Calibrators were prepared for measurement as described above and used within 30 minutes of preparation. Calibration was performed once per reagent lot and as required for maintaining QC values within specified limits (instrument specifications). The lot calibration stability period is 28 days.

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The QC material, PreciControl Everolimus, at 3 concentration levels, was provided by Roche Diagnostics. The QC samples were prepared and stored using the same methodology as for the calibrators. Once prepared, QC samples were measured within 30 minutes. Each instrument run was validated by measuring the QC material and comparing control results with specified ranges before patient sample material was measured.

The measuring range of the everolimus ECLIA is 0.5–30 mcg/L (defined by the LLoQ without considering bias and the maximum of the master curve).[9] Values below the LLoQ without considering bias are reported as <0.5 mcg/L and those above the measuring range are reported as >30 mcg/L.

The QMS Everolimus assay (Thermo Fisher Scientific) is a homogeneous particle-enhanced turbidimetric immunoassay with a measuring range of 2.0–20.0 mcg/L (according to the assay documentation, with the lower value based on the lower limit of quantitation).[11] The lower limit of detection for the QMS assay has been reported as 0.7 mcg/L.[12] The QMS Everolimus assay procedures were performed according to the manufacturer's instructions on a Thermo Fisher Scientific Indiko Plus analyzer.[11] This included the use of the value-assigned calibrators and controls from the manufacturer to minimize the positive bias from the QMS assay against LC-MS/MS.[5,11–15]

Each investigational site performed LC-MS/MS based on their protocols developed and routinely used at the site. Table 1 summarizes LC-MS/MS methods used by each laboratory.

Inaccuracy

The ECLIA was used to measure PC E (n = 3) and IPT (n = 6) samples on either the *cobas e* 411 or *cobas e* 601 analyzers. Samples were also measured using LC-MS/MS methods at all centers, and with the QMS assay at the center in Ghent.

Calibration was performed on the LC-MS/MS as a part of each run, as described above for the ECLIA. Samples were divided into 3 aliquots and measured in a single run with 3 replicates for each sample. Inaccuracy, expressed as relative mean recovery (%) using the target value as conventional value, was calculated for each sample in each center. LC-MS/MS target values for the IPT samples were provided by LGC Standards and based on the mean values assigned by the proficiency testing program. The results from these proficiency tests were used as the reference values in this evaluation.

Imprecision

The repeatability (within-run) and intermediate (within-lab) imprecision experiments according to the CLSI EP05-A3 guideline were performed at 4 sites (Stuttgart, Brussels, Ghent, and Barcelona).[16] A total of 84 aliquots from each of the 3 PC E controls and the 5 HSP samples were tested over a 21-day period using a model with 2 runs per day and 2 replicates per run. Samples were randomized for each run.

Reproducibility was assessed at 3 centers (Stuttgart, Ghent, and Munich) according to CLSI EP05-A3 guidelines.[16] Site-to-site, lot-to-lot, and day-to-day variance components were estimated as well as their sum (reproducibility). Each site used 2 different reagent lots, and samples were measured over 5 days, with 5 aliquots per sample (crossed-nested design), resulting in 150 measurements from each sample. The same 3 PC E samples and 5 HSP samples used to measure repeatability and intermediate imprecision were also used for the reproducibility experiments. Protocol-defined acceptance criteria for repeatability were ≤ ±0.24 mcg/L (SD) for everolimus concentrations in the range 0.5–3 mcg/L and coefficient of variation (CV) of ≤8% for concentrations >3–30 mcg/L. For intermediate imprecision, the acceptance criteria were ≤ ± 0.360 mcg/L (SD) for levels in the range of 0.5–3 mcg/L and CV of ≤12% for concentrations >3–30 mcg/L. The acceptance criteria for reproducibility were ≤ ±0.54 mcg/L (SD) for everolimus in the range of 0.5–3 mcg/L and ≤18% (CV) for concentrations >3–30 mcg/L. The acceptance criteria were determined based on discussions with laboratory users.

Lower Limit of Quantification

The LLoQ without considering bias of the everolimus ECLIA was determined by assessing the lowest concentration that could be estimated with a CV ≤20%. The ECLIA LLoQ without considering bias was assessed at the Munich site using the *cobas e* 411 analyzer. Eleven HSP samples (described previously, 1 aliquot per sample) were analyzed in each run; 2 runs were performed per day for 5 days in total.

Interchangeability Studies (Method Comparisons)

Method comparisons were performed to assess whether the new Elecsys Everolimus ECLIA was interchangeable with the LC-MS/MS methods and the QMS Everolimus assay. Method comparison experiments with LC-MS/MS methods were performed at all centers and additionally with the QMS assay at the center in Ghent. Each study center used anonymized residual samples and aimed to test native samples from at least 60 kidney, 60 liver, and 60 heart transplant recipients. In total, 200 native samples were tested in Barcelona, 70 in Brussels, 153 in Ghent, 177 in Munich, and 184 in Stuttgart using different LC-MS/MS methods and the ECLIA. In addition, in Ghent, 151 native samples were processed using the QMS assay and using the ECLIA.

Statistical Analysis

The everolimus ECLIA test results were captured directly from a laptop computer attached to the *cobas e* 411/c 6000 analyzer using the Windows-based Computer-Aided Evaluation (WinCAEv) program, version 2.2.2, CFR 21 Part 11–compliant electronic data capture software that had been developed and validated for Roche-sponsored studies.[17] Reference assay output was entered offline into WinCAEv at each center and source data verified from analyzer printouts.

For imprecision, the mean, SD, and CV were estimated using statistical software R (The R Foundation) with the variance component analysis package version 1.2.1.[18] For reproducibility experiments, a variance component analysis

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TABLE 1. LC-MS/MS Methods Used at the 5 Investigational Sites

Investigational Center	LC/MS Manufacturer/ Model	Method Working Range (mcg/L)	Sample Preparation (Extraction)	Internal Standard	Calibrators	Chromatographic Separation	Interday Imprecision Values (CV%)
Barcelona	Waters Acquity UPLC/ Waters TQD	1.42–49.5	PPT with $ZnSO_4$ and acetonitrile	$[^{13}C_2D_4]$-everolimus	RECIPE ClinCal (whole blood calibrator set level 0–6)	Column: Acquity UPLC BEH™ C18 reverse-phase column (30 × 2.1 mm) (Waters, Milford, MA); Mobile phase: A: 0.1% (v/v) formic acid and 2 mmol/L ammonium acetate in water, B: 0.1% (v/v) formic acid and 2 mmol/L ammonium acetate in methanol; Elution Mode: nonlinear gradient. Temperature: 55°C; Injected sample volume: 20 μL; Run time: 2.5 min	≤8.7
Brussels	Agilent 6460 + HPLC Agilent 1290	1.2–45.0	$ZnSO_4$ precipitation with methanol	everolimus d4	RECIPE ClinCal (whole blood calibrator set level 0–6)	Column: Agilent Zorbax C18, 4.6 × 50 mm	≤3.89
Ghent	Waters Acquity UPLC/ Waters TQD	0.5–30.0	PPT with $ZnSO_4$ and acetone followed by liquid–liquid extraction with chlorobutane	32-dimethoxy-rapamycin (Wyeth # AY-24668-1)	Chromsystems whole blood calibrator	Column: Waters MassTrak TDM C18 2.1 × 10 mm; Mobile Phase: 2 mmol/L ammonium acetate +0.1% formic acid in water and methanol; Elution mode: gradient 50%–100%; Temperature: 55°C; Injected sample volume: 20 μL; Run time: 1.8 min	≤7.9
Munich	Waters Quattro Ultima Pt	0.6–50	PPT with MeOH/ $ZnSO_4$ (4:1) followed by on-line solid phase extraction (Oasis HLB trapping column)	$[^{13}C_2D_4]$-everolimus	Chromsystems 6PLUS1	Column: Waters Sunfire C18 column. Ninety percent MeOH/ 10% 0.1% formic acid, isocratic elution	≤9.5

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TABLE 1. (*Continued*) LC-MS/MS Methods Used at the 5 Investigational Sites

Investigational Center	LC/MS Manufacturer/ Model	Method Working Range (mcg/L)	Sample Preparation (Extraction)	Internal Standard	Calibrators	Chromatographic Separation	Interday Imprecision Values (CV%)
Stuttgart	Waters H-Class Acquity/ Xevo-TQD	0.6–50	PPT with $ZnSO_4$ and acetonitrile	everolimus d4	Chromsystems 6PLUS1	Column: MZ-Analysentechnik MZ Aqua Perfect C18 150 × 3.0 mm, 5 μm; Mobile phase: A: 0.1% (v/v) formic acid and 2 mmol/L ammonium acetate in water, B: 0.1% (v/v) formic acid and 2 mmol/L ammonium acetate in methanol; Elution Mode: nonlinear gradient; Temperature: 65°C; Injected sample volume: 10 μL; Run time: 5.0 min	≤7.10

PPT, protein precipitation.

was performed to breakdown the overall variance into single factors contributing to the total observable variability (multi-lot reproducibility), which corresponded to the sum of all individual variance components (site-to-site, lot-to-lot, day-to-day, and repeatability). Imprecision results and outliers were handled in compliance with the CLSI EP05-A3 guidelines.[16] For the repeatability and intermediate imprecision assessments, it was permitted to reject at most 2 results because of an outlier. This rule was applied for each imprecision sample and each 21-day experiment and did not include runs eliminated due to failed QC or handling errors (wrong sample, missing values, etc). In these cases, the whole day was excluded and replaced by another. For reproducibility experiments, it was permitted to reject at most 1 result because of an outlier, and this was applied for each precision sample and each 5-day experiment.

Method comparison evaluation was performed with WinMC 2.0 using exported data from WinCAEv and was compliant with the CLSI EP09-A3 guidelines.[19] Weighted Deming regression analysis was used for method comparisons with each center's LC-MS/MS method and with the QMS Everolimus assay. Pearson's correlation coefficient (r) was calculated for each comparison. To gain further information on method comparability, Bland–Altman difference plots were performed.[20] The relative bias at clinically relevant medical decision points was determined for the everolimus ECLIA versus LC-MS/MS method comparisons. The medical decision points were defined as the therapeutic range for everolimus, which was between 3 and 8 mcg/L.

RESULTS

Inaccuracy

With respect to the 3 PC E samples, at concentrations of 2.71, 9.84, and 20.1 mcg/L, all everolimus ECLIA results were within a mean % recovery range of 91.0%–105.4% (Table 2); the median bias across the analytical sites was −1.4% to +2.6%. The mean % recovery range for the LC-MS/MS was 81.1%–130.3% (median bias, −0.8% to +10.4%), whereas, the recovery ranges relative to target values were markedly lower for the QMS Everolimus assay (57.6%–72.5%); however, this was performed at 1 site only. This finding is most likely explained by standardization of the assay against patient samples, which usually show high levels of metabolite cross-reactivity that cannot be observed in spiked QC materials.

With respect to the spiked IPT samples, the everolimus ECLIA results generally fell within the mean % recovery range of 78.5%–122.8% with 1 exception (IPT 73A). All LC-MS/MS methods were within the mean % recovery range of 90.9%–128.4%. Differences in recovery rates were not unexpected because each center used different calibrators (2 centers used RECIPE calibrators and 3 centers used Chromsystems calibrators), chromatographic conditions, and mass-spectrometer setup. Recovery rates using the QMS everolimus assay relative to target were markedly lower (range from 54.9% to 87.2%), a finding that mirrored the results observed using the PC E samples.

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TABLE 2. Mean Measured Values (mcg/L) and the Mean Recovery Rate Relative to Target Value (%) in PC E and IPT Samples

	Investigational Center						
	Ghent			Stuttgart		Munich	
	ECLIA	LC-MS/MS	QMS	ECLIA	LC-MS/MS	ECLIA	LC-MS/MS
Samples (Target Concentration)	Mean Concentration Measured, mcg/L (Mean % Recovery Relative to Target Value)						
PC E L1 (2.71 mcg/L)	2.47 (91.3)	2.87 (105.8)	1.57 (57.8)	2.73 (100.7)	2.69 (99.2)	2.72 (100.3)	2.43 (89.8)
PC E L2 (9.84 mcg/L)	9.06 (92.0)	11.07 (112.5)	5.67 (57.6)	9.77 (99.3)	9.88 (100.4)	9.74 (99.0)	9.30 (94.5)
PC E L3 (20.1 mcg/L)	19.76 (98.3)	24.50 (121.9)	14.57 (72.5)	20.79 (103.4)	22.00 (109.5)	21.18 (105.4)	20.87 (103.8)
IPT 73A (1.1 mcg/L)	1.21 (110.3)	1.00 (90.9)	N/a*	1.80 (163.4)	1.21 (109.7)	1.54 (139.7)	1.23 (112.1)
IPT 73C (3.0 mcg/L)	3.08 (102.7)	3.07 (102.2)	1.65 (54.9)†	3.68 (122.8)	3.18 (105.9)	3.03 (101.0)	3.27 (108.9)
IPT 76A (5.0 mcg/L)	4.74 (94.7)	5.50 (110.0)	4.36 (87.2)	6.07 (121.3)	5.42 (108.5)	5.30 (105.9)	5.60 (112.0)
IPT 76C (10.2 mcg/L)	9.35 (91.6)	11.27 (110.5)	7.86 (77.0)	11.26 (110.4)	10.42 (102.1)	9.91 (97.2)	11.47 (112.4)
IPT 77C (10.0 mcg/L)	9.29 (92.9)	11.53 (115.3)	8.26 (82.6)	11.28 (112.8)	10.32 (103.2)	9.44 (94.4)	11.27 (112.7)
IPT 78A (14.4 mcg/L)	12.13 (84.3)	14.80 (102.8)	11.92 (82.8)	14.76 (102.5)	15.16 (105.3)	11.31 (78.5)	16.13 (112.0)

	Investigational Center				All Sites	
	Brussels		Barcelona		Median Measured Concentration (Median % Recovery) Across All Sites	
	ECLIA	LC-MS/MS	ECLIA	LC-MS/MS	*cobas e* 411	LC-MS/MS
Samples (Target Concentration)	Mean Concentration Measured, mcg/L (Mean % Recovery Relative to Target Value)					
PC E L1 (2.71 mcg/L)	2.49 (91.8)	2.45 (90.2)	2.72 (100.4)	3.40 (125.5)	2.72 (100.3)	2.69 (99.2)
PC E L2 (9.84 mcg/L)	9.71 (98.6)	7.98 (81.1)	9.32 (94.7)	11.77 (119.6)	9.71 (98.6)	9.88 (110.4)
PC E L3 (20.1 mcg/L)	20.63 (102.6)	20.37 (101.4)	18.29 (91.0)	26.2 (130.3)	20.64 (102.6)	22.0 (109.5)
IPT 73A (1.1 mcg/L)	1.67 (152.1)	1.35 (122.7)	1.47 (133.6)	1.30 (118.2)	1.54 (139.7)	1.23 (112.1)
IPT 73C (3.0 mcg/L)	2.91 (96.9)	3.12 (104.1)	3.08 (102.8)	3.57 (118.9)	3.08 (102.7)	3.18 (105.9)
IPT 76A (5.0 mcg/L)	5.42 (108.3)	5.42 (108.5)	5.13 (102.5)	6.10 (122.0)	5.30 (105.9)	5.50 (110.0)
IPT 76C (10.2 mcg/L)	10.01 (98.2)	11.02 (108.1)	9.15 (89.7)	13.10 (128.4)	9.91 (97.2)	11.27 (110.5)
IPT 77C (10.0 mcg/L)	9.78 (97.8)	11.61 (116.1)	10.01 (100.1)	12.37 (123.7)	9.78 (97.8)	11.53 (115.3)
IPT 78A (14.4 mcg/L)	13.50 (93.8)	16.03 (111.3)	13.31 (92.4)	18.20 (126.4)	13.31 (92.4)	16.03 (111.3)

*3/3 of these results were lower than the measuring range of the assay (all were <1.5 mcg/L).
†1/3 results were lower than the measuring range (all were <1.5 mcg/L); therefore, this value was calculated based on 2/3 results.

Imprecision

Repeatability and Intermediate Imprecision

With respect to repeatability estimated from pooling all 4 sites, the SDs of the lower everolimus concentration control samples (PC E L1 and HSP 2; 0.5–3.0 mcg/L) were 0.142 and 0.108 mcg/L, respectively, and CVs were all ≤4.8% for the higher concentration samples and were thus within the predefined acceptance criteria (Table 3). Intermediate imprecision SDs were ≤0.263 mcg/L for the lower concentration samples, and the CVs were ≤8.4% for the rest of the processed samples, which again were within the predefined acceptance criteria.

Reproducibility

Lot-to-lot and site-to-site variability SDs were all ≤0.15 mcg/L for sample concentrations in the range 0.5–3 mcg/L (HSP2 samples) and ≤7.0% CV for samples

TABLE 3. Repeatability and Intermediate Imprecision of the Everolimus ECLIA Obtained in 4 Centers (Pooled Data)

Sample (Target Concentration) mcg/L	Mean Concentration, mcg/L (Measured Range)	Repeatability, SD/CV (Measured Range)	Intermediate Imprecision, SD/CV (Measured Range)
PC E L1 (2.71)	2.78 (2.55–3.18)	0.14 mcg/L (0.09–0.17)	0.26 mcg/L (0.22–0.36)
PC E L2 (9.84)	9.91 (9.34–10.59)	3.1% (2.5–3.5)	5.6% (4.8–6.5)
PC E L3 (20.1)	20.95 (20.03–21.20)	3.3% (2.6–3.8)	5.9% (4.4–6.9)
HSP2 (0.5–3)	2.39 (2.14–2.75)	0.11 mcg/L (0.08–0.12)	0.23 mcg/L (0.16–0.32)
HSP3 (3–8)	5.60 (5.24–6.20)	4.6% (3.8–5.6)	8.4% (5.7–11.3)
HSP4 (8–16)	11.97 (11.38–12.73)	4.0% (3.1–4.6)	5.9% (5.5–6.4)
HSP5 (16–24)	20.54 (19.83–21.38)	4.8% (2.3–7.9)	7.9% (4.5–12.2)
HSP6 (24–30)	24.89 (24.82–25.27)	4.1% (3.3–5.1)	7.4% (4.9–8.3)

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TABLE 4. Reproducibility of the Everolimus ECLIA at 3 Centers Based on 150 Measurements of Each Sample

Sample (Target Concentration) mcg/L	Mean Measured Conc., mcg/L	Lot-To-Lot (SD/CV)	Site-To-Site (SD/CV)	Multi-Lot Reproducibility† (SD/CV)
PC E L1 (2.71)	3.06	<0.1%	7.0%	13.7%
PC E L2 (9.84)	10.22	<0.1% (<0.1%)*	5.6%	9.7%
PC E L3 (20.1)	21.27	<0.1% (<0.1%)*	5.6%	9.8%
HSP2 (0.5–3.0)	2.60	0.00 mcg/L	0.15 mcg/L	0.34 mcg/L
HSP3 (3.0–8.0)	5.85	<0.1%	6.6%	12.4%
HSP4 (8.0–16.0)	12.22	<0.1% (<0.1%)*	4.8% (4.5%)*	8.7% (8.8%)*
HSP5 (16.0–24.0)	20.94	<0.1% (<0.1%)*	4.8% (5.1%)*	8.9% (9.2%)*
HSP6 (24.0–30.0)	25.25	<0.1% (<0.1%)*	5.8%	10%

*Initial value with outlier.
†Multi-lot reproducibility, total observable variability that corresponds to the sum of all individual variance components (site-to-site, lot-to-lot, and day-to-day repeatability).

of concentrations >3–30 mcg/L (PC E L1–3 and HSP3–6 samples) (Table 4). These results were based on 150 measurements of each sample at the 3 centers and were within predefined acceptance criteria. This held true when outliers, defined according to CLSI EP05-A3 criteria, were also included in the analyses.

In terms of the multi-lot reproducibility for the samples with low everolimus concentrations, CV for the PC E L1 sample was 13.7% at a measured mean concentration of 3.06 mcg/L. The SD for the HSP2 sample was 0.338 mcg/L at a measured mean concentration of 2.60 mcg/L. The CVs for the higher concentration samples (PC E L2–3 and HSP3–6 samples) were all ≤12.4%.

LLoQ Without Bias

Figure 1 shows a plot of CVs determined from a single measurement of 8 evaluable pools (3 of the 11 samples fell below 0.3 mcg/L everolimus and so were not included) over 10 runs in 5 days (2 runs per day) at each concentration. The CV at the LLoQ without considering bias (0.5 mcg/L) was 9.3%.

Interchangeability Studies (Method Comparisons)

Weighted Deming regression analysis of the everolimus ECLIA results versus LC-MS/MS results, based on ethylenediaminetetraacetic acid whole blood samples from all 784 transplant patients, yielded a slope of 1.21 [95% confidence interval (CI): 1.15–1.26], intercept of 0.478 mcg/L (95% CI: 0.241–0.716), and Pearson's r of 0.91 (Fig. 2A). Pooled data sets for each organ type (kidney, liver, and heart) were comparable in that the slope did not differ significantly between samples from different organs. Data from liver transplant patients showed a wider scatter than data from kidney or heart transplant patients, possibly due to impaired hepatic metabolism of everolimus in liver transplant patients resulting in a potentially more complex, heterogeneous, and pronounced metabolite profile. For completeness, the method comparison results at the individual sites are shown in **Supplemental Digital Content 1** (see **Supplementary Figure 1**, http://links.lww.com/TDM/A226).

Bland–Altman analysis revealed that the mean bias for whole blood samples measured on the ECLIA versus LC-MS/MS methods was 1.46 mcg/L (±2 SD −0.98 to +3.91; Fig. 2B). The estimated relative bias at the medical decision points for everolimus (ie, at the lower and upper limits of the therapeutic range, 3–8 mcg/L) were 36.5% (95% CI: 33.6%–39.3%) at 3 mcg/L and 26.5% (95% CI: 23.4%–29.4%) at 8 mcg/L.

Comparison of the results obtained by everolimus ECLIA and by the QMS everolimus assay yielded a slope of 1.05 (95% CI: 0.917–1.17) (Fig. 2C), intercept of 1.03 mcg/L (95% CI: 0.351–1.70), and Pearson's r of 0.91. The mean bias calculated by Bland–Altman analysis was 1.23 mcg/L (±2 SD −1.37 to +3.82; Fig. 2D).

DISCUSSION

This multicenter evaluation of the analytical performance characteristics of the everolimus ECLIA showed that this new immunoassay meets the performance criteria defined in recently published guidelines for routine TDM of everolimus in patients undergoing solid organ transplantation, with respect to method imprecision and inaccuracy.[5]

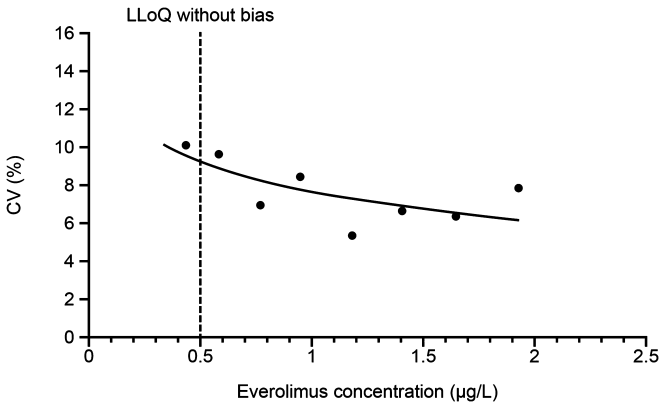

FIGURE 1. Determination of LLoQ without considering bias of the everolimus ECLIA, using HSP samples across a target concentration range of 0.2–2.0 mcg/L. LLoQ without considering bias was defined as 0.5 mcg/L.

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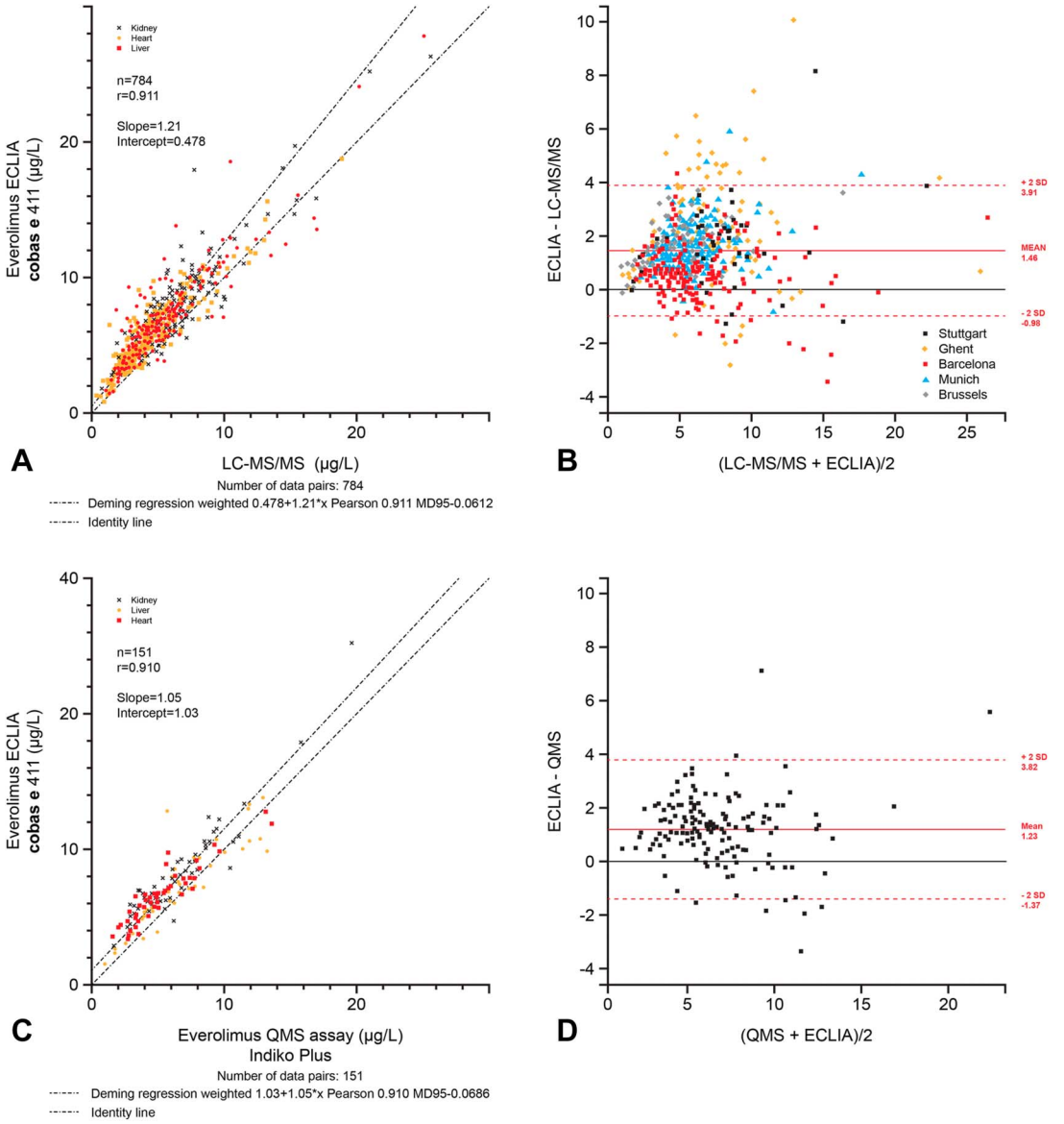

FIGURE 2. Weighted Deming regression and Bland–Altman plots of the everolimus ECLIA versus LC-MS/MS methods (A, B) and versus QMS everolimus assay (C, D), using native samples from solid organ transplant patients.

According to international recommendations, a CV below 5% (at middle and high therapeutic concentrations) and better than 10% (for concentrations at the lower end of the therapeutic range) should be achieved with assays for immunosuppressive drugs.[15,21] Results from this study showed that the everolimus ECLIA met these criteria. At least 95% of all measured data for repeatability and intermediate imprecision were within internationally recognized acceptance criteria; intermediate imprecision found at subtherapeutic concentrations of ~2.8 mcg/L was <6% (the therapeutic range of everolimus is estimated to be 3.0–8.0 mcg/L),[6] highlighting the suitability of this assay for clinical practice.

The ECLIA had an LLoQ without considering bias of 0.5 mcg/L with a CV of 9.3%. This emphasizes that the measurement range of the everolimus ECLIA is appropriate for the span of drug concentrations observed in samples typically seen in routine clinical practice, as well as for extended pharmacokinetic investigations.

Procedures with a high detection capability are needed for TDM of everolimus because of the relatively low whole blood concentrations obtained with the doses of everolimus used for immunosuppression.[13] Despite their complexity, LC-MS/MS methods have been widely adopted because they possess the requisite blend of performance characteristics including capability of detection, selectivity, precision, and accuracy. Recently published guidelines suggest that, at present, a fully validated LC-MS/MS method should be considered the preferred method for measuring trough concentrations of everolimus in whole blood.[5,13] Therefore, each center's LC-MS/MS method was used as the reference

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method in this study for comparative purposes. Weighted Deming regression analysis of the everolimus ECLIA results, in comparison with those obtained using LC-MS/MS methods, generally demonstrated a linear correlation between the 2 test procedures when based on pooled results from all 5 centers. To meet recent consensus guidelines for the selective measurement of everolimus concentrations, the linear regression slope should be within ±10% of the theoretical value of 1.0 together with a linear regression intercept that is not statistically significantly different from zero and a standard error of the estimate (S_{yx}) ≤10% of the average of the therapeutic concentrations.[5,13] Our results, using native samples, suggest that these tighter criteria were not met because the slope for the pooled data exceeded 1.1. The magnitude of the slope deviation was consistent with that reported in a recent single-center method comparison study of the Elecsys everolimus assay (*cobas e* 411 analyzer; Roche Diagnostics GmbH, Rotkreuz, Switzerland) and LC-MS/MS methodology, where the slope was 1.131 (Passing–Bablok regression).[22] Immunoassays can be affected by cross-reactivity, especially from metabolites of the parent drug, which can lead to overestimation of drug concentrations and potential for dosing inaccuracies.[23] The higher slope value shown in our study was likely due to cross-reactivity of metabolites. This slope/deviation bias suggests that individual patients should be rebaselined when switching from LC-MS/MS method to the ECLIA. However, rebaselining is also required when switching from LC-MS/MS to a different LC-MS/MS method due to lack of interchangeability between the methods. Redefinition of the target ranges for everolimus would also be necessary.[5,6,22]

The QMS everolimus assay is one of the more recently approved tests for quantitation of everolimus concentrations in whole blood in organ-transplanted patients.[12,24] In our comparison between the everolimus ECLIA results and the QMS everolimus assay results, on samples from solid organ transplant recipients, good agreement was observed between the 2 assays with respect to the slope value and Pearson correlation coefficient. However, there was also an intercept of 1.03, meaning bias; therefore, interchangeability was not demonstrated. It should be noted that method comparisons between the QMS assay and the LC-MS/MS method revealed a significant positive bias with the QMS everolimus assay, such that 69% of whole blood samples from organ transplant recipients were at supratherapeutic concentrations.[11,12,14,15] This overestimation of everolimus concentrations could result in inadequate dose adjustments in clinical practice, as previously reported.[15,22] Therefore, to minimize the bias between the QMS everolimus assay and the LC-MS/MS method, the QMS everolimus calibrators and QCs from the manufacturer are initially value assigned using a representative set of clinical trough samples from renal and liver transplant patients with traceability to LC-MS/MS values, which are 70% of their gravimetric concentrations.[5,11,13] Again, the present findings are in agreement with those from a recent single-center study, which demonstrated that results generated using the Elecsys everolimus assay (*cobas e* 411 analyzer), QMS everolimus assay (Dimension Xpand Plus analyzer; Siemens Healthcare GmbH, Erlangen, Germany), and LC-MS/MS methodology were not consistent regarding their diagnostic value.[22] Compared with the earlier study,[22] this study is novel in several respects, including its multicenter design; the evaluation of the accuracy, imprecision (repeatability, intermediate imprecision, and reproducibility), and LLoQ without considering bias of the Elecsys assay; demonstration of the interchangeability between results obtained using the everolimus ECLIA and by 5 different LC-MS/MS methods (based on the analysis of 784 native samples in total); and interchangeability between results obtained using the everolimus ECLIA and QMS assays applied to the Thermo Fisher Scientific Indiko Plus analyzer.

Although the LC-MS/MS method is considered the current method of choice for monitoring trough concentrations of everolimus in solid organ transplant recipients, the everolimus ECLIA offers practical advantages over LC-MS/MS methods, especially with respect to ease of performance and time taken to perform analyses. The use of ECLIA technology does not impose any limitations on the development of new assays. For example, the technology allows for the development of a wide range of assay types, ranging from small molecules (eg, steroid hormones) to very large proteins (eg, thyroglobulin), and also offers additional advantages over existing methods, including a rapid turnaround time and long reagent stability.[25,26]

CONCLUSIONS

In this large multicenter evaluation, the everolimus ECLIA demonstrated good precision, accuracy, and sensitivity for TDM of everolimus in solid organ transplant recipients and generally agreed with LC-MS/MS methods. However, in some centers, there was evidence of slope deviation/bias in the method comparison experiments, suggesting that it may be necessary to rebaseline individual patients when switching from LC-MS/MS methodology to ensure proper patient handling.

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