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Multicenter Analytical Evaluation of the Automated Electrochemiluminescence Immunoassay for Cyclosporine

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Background: Cyclosporine A (CsA) is used as a posttransplantation immunosuppressant drug, and careful monitoring of CsA concentration in whole blood is essential. A new automated electrochemiluminescence immunoassay (ECLIA) for CsA measurement has been assessed in a multicenter evaluation.

Methods: Residual EDTA whole blood samples from patients undergoing CsA therapy after organ transplant were used in assay evaluation at 5 clinical laboratories in Europe. Experiments included imprecision according to CLSI EP5-A2 (within-run and intermediate), lower limit of quantification, linearity according to CLSI EP6-A, and recovery of commercial external quality control samples. In addition, comparisons to liquid chromatography-tandem mass spectrometry methods in routine use at each investigational site and to commercial chemiluminescent microparticle immunoassay and antibody-conjugated magnetic immunoassay methods were performed.

Results: Imprecision testing gave coefficients of variation of less than 9% in the 30–2000 mcg/L range for both within-run and intermediate imprecision. Lower limit of quantification of 6.8 mcg/L at one investigational site and 1.8 mcg/L at a second site at 20% coefficient of variation were observed. Linearity was measured over the concentration range 0–2000 mcg/L, yielding a deviation of less than ±12%. External quality control sample recovery by ECLIA was 93%–114% of LC-MS/MS sample recovery. Deming regression analysis of ECLIA method comparison to combined LC-MS/MS results yielded a slope of 1.04 [95% confidence interval (CI), 1.03–1.06] and intercept of 2.8 mcg/L (95% CI, 1.5–4.1 mcg/L). Comparison to chemiluminescent microparticle immunoassay yielded a slope of 0.87 (95% CI, 0.85–0.89) and intercept of 1.4 mcg/L (95% CI, −0.89 to 3.7 mcg/L); comparison to antibody-conjugated magnetic immunoassay yielded a slope of 0.96 (95% CI, 0.93–0.98) and intercept of −4.2 mcg/L (95% CI, −7.1 to −1.2 mcg/L).

Conclusions: The data from this multicenter evaluation indicate that the new ECLIA-based cyclosporine assay is fit for its purpose, the therapeutic monitoring of CsA.

Key Words: cyclosporine, immunoassay, therapeutic drug monitoring

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INTRODUCTION

Cyclosporine A (Ciclosporin, Cyclosporin, CsA) was introduced in the early 1980's to transplantation medicine as the first calcineurin inhibitor. Replacing azathioprine, the compound became the mainstay of long-term immunosuppressive therapy during the following years. It was soon recognized that due to variable enteral absorption and extensive metabolism (also modulated by a number of xenobiotic compounds), therapeutic drug monitoring is indispensable for immunosuppressive therapy with CsA. Consequently, a number of immunoassays and chromatographic analytical methods for monitoring CsA have been introduced over the years.[1–4]

Beginning in 2000, a second calcineurin inhibitor, tacrolimus, became more and more widely used. Since then, tacrolimus has been replacing CsA as the first-line immunosuppressant in most institutions. The results of the Symphony study, published in 2007, have encouraged this trend.[5] However, CsA is still a key drug in transplantation medicine. In a substantial number of stable long-term transplantation patients, CsA-based regimens are continued today. The decision to change a patient's therapy from tacrolimus to CsA may be made in situations where specific side effects of tacrolimus occur.[6] Furthermore, there is debate about a higher incidence

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of diabetes being observed under tacrolimus therapy as compared with CsA.[7–11] In addition, CsA is the only immunosuppressant approved for the treatment of graft-versus-host disease after bone marrow transplantation. From a global perspective, the lower therapy costs of CsA as compared with tacrolimus are also relevant.

At present, the global share of CsA in transplantation medicine is about 20%. In several of the laboratories involved in this multicenter evaluation (MCE), 15%–20% of requests for immunosuppressant monitoring are presently for CsA (15% in Munich, 19% in Ghent, and 15%–20% in Brussels). Therefore, the need remains for a robust and time-efficient CsA assay to fulfill the therapeutic monitoring needs of the clinical routine laboratory.

External Quality Assessment Schemes provide insight into performance and prevalence of diagnostic assays in participating laboratories. Analytical Services International administers the Ciclosporin International Proficiency Testing Scheme (IPTS). IPTS is available for several immunosuppressive drugs and can include samples that are spiked to a known concentration and pooled patient samples.[12,13] Quantification of CsA in blood using LC-MS/MS has become more common in clinical laboratories in recent years; however, in the January 2014 (Cic358) report from the Ciclosporin IPTS, almost 65% of the results reported are from measurements made with immunoassays. The chemiluminescent microparticle immunoassay (CMIA) and the antibody-conjugated magnetic immunoassay (ACMIA) account for approximately 40% of the immunoassay results in the January 2014 Ciclosporin IPTS report. These data from the world's largest CsA proficiency testing scheme can be considered as representative of the global demand for immunosuppressant assays outside of the United States. In the United States, the assay distribution is somewhat different. According to the College of American Pathologists external Proficiency Testing program report for CsA from 2008, approximately 85% of program participants used immunoassays and approximately 75% of participants used the CMIA immunoassay. This suggests that implementation of LC-MS/MS methods is still a major challenge for the clinical routine laboratory. However, the number of participating laboratories in the proficiency testing programs does not necessarily reflect the number of patients monitored by different methods. Indeed, laboratories that infrequently perform CsA tests may be less likely to establish and maintain an LC-MS/MS method.

Automated immunoassays for immunosuppressive drug monitoring are clearly important for the modern clinical routine laboratory. A new automated electrochemiluminescence immunoassay (ECLIA) for the quantification of CsA in whole blood has been developed to be implemented on an existing commercial analyzer. Thorough evaluation of this test is of importance for the transplantation medicine community. The aim of this MCE involving 5 clinical laboratories in Europe was to examine reproducibility, lower limit of quantification (LLOQ), linearity, and agreement of results between the new assay and other methods that are currently in use.

MATERIALS AND METHODS

An MCE of the ECLIA assay was conducted from October 2012 to April 2013 at 5 European investigational sites in Germany (Klinikum-Stuttgart, Stuttgart and Hospital of the University of Munich, Munich), Belgium (University Hospital St. Luc, Brussels and Ghent University Hospital, Department of Laboratory Medicine, Ghent), and Spain (Laboratori Clinic Department, Hospital Universitari de Bellvitge, Barcelona). Waivers were obtained from the Ethics Committees of the respective institutions, when remnant samples from the institution were used, before clinical study work began. Blood samples evaluated at the Ghent site were shipped frozen on dry ice from other investigational sites. In addition, all investigational sites conducted the study in accordance with the Declaration of Helsinki (rev. Tokyo, Venice and Hong Kong) and following ICH Good Clinical Practice guidelines.

Immunoassays

The ECLIA (Elecsys Cyclosporine; Roche Diagnostics GmbH, Mannheim, Germany) assay for use on the cobas e analyzer (Roche Diagnostics) uses the principle of electrochemiluminescence for measurement[14] (Fig. 1). The development of ECLIA is based on the use of a ruthenium complex and tripropylamine. The materials to be measured [calibrators, quality control (QC) material, or patients' whole blood samples] are equilibrated to room temperature (18–25°C) and mixed without vortexing directly before use to resuspend any

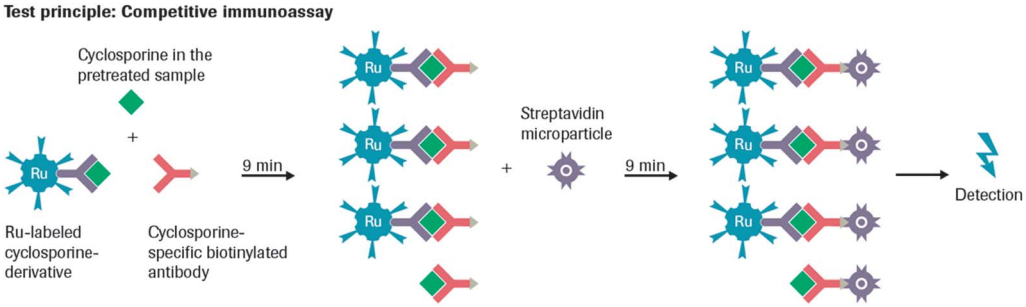


FIGURE 1. Schematic representation of the ECLIA measuring principle. Pretreated samples are combined with biotinylated analyte-specific antibodies and labeled analyte derivatives and recovered with streptavidin microparticles.[15] Detection is by electrochemiluminescence (see Materials and Methods).

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sedimented erythrocytes. Three hundred microliters of the material to be measured is combined with 300 μL of Elecsys ImmunoSuppressive Drug (ISD) Sample Pretreatment Reagent (Roche Diagnostics), a methanol-based solution containing zinc sulfate, in a microcentrifuge tube. The tube is capped and vortexed for at least 10 seconds. The samples are then centrifuged for 4 minutes in a microcentrifuge at ≥10,000*g*. The supernatant is decanted into a Hitachi sample cup and capped until loading onto the system. Once in the analyzer, in a first 9-minute incubation step, 20 μL of pretreated sample is incubated with a CsA-specific biotinylated antibody and a ruthenium complex-labeled CsA derivative. Formation of the respective immune complex depends on the CsA concentration in the sample. The antibody binding site is occupied partially with CsA from the sample and partially with ruthenylated hapten. In the next step, streptavidin-coated magnetic microparticles are added. During the second 9-minute incubation, the entire complex becomes bound to the solid phase through interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are removed by a washing step, and application of voltage to the electrode induces chemiluminescent emission, which is measured by a photomultiplier.[15] According to the competitive assay design, an inverse proportional calibration curve is obtained.

Manufacturer reference standardization for the ECLIA assay was carried out using CsA reference material (Novartis, Switzerland). CsA reference material was analyzed according to testing monograph DS 3142056_R_02, which specifies identical requirements as defined for CsA USP material. CsA with its defined analyte content is dissolved in a defined quantity of human whole blood. This stock solution is used to manufacture a set of reference calibrators by spiking human whole blood. The CsA concentration of the working calibrators and of a panel of human samples is determined using this calibration curve. The CsA concentration values for the working calibrators obtained in this manner represent the end of the reference standardization and make it possible to calibrate the ECLIA assay and to establish the particular typical Rodbard curve parameters. Finally, in an additional value assignment, the CsA concentrations for the product 2-point calibrators are established. For each product kit used at the investigational sites, the ECLIA assay was calibrated using the Calset Elecsys Cyclosporine (Roche Diagnostics) with 2 concentrations (typical values are 35 and 1540 μg/L). Calibrators were reconstituted according to the manufacturer's instructions and stored in 300 μL aliquots at 2–8°C or below −15°C. Calibrators were prepared for measurement as described above and used for calibration 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.

Sample measurement using CMIA (ARCHITECT CYCLOSPORINE; Abbott Laboratories, IL) on the Abbott Architect platform and ACMIA [Dimension Cyclosporine (CSA), Siemens Healthcare Diagnostics, Inc., IL] on the Siemens Dimension platform were performed according to the manufacturers' instructions and according to site-specific standard operating procedures. Measurements using CMIA at the site in Stuttgart were performed at the Marienhospital, Stuttgart. Calibration of the CMIA and ACMIA assays on their respective platforms were also performed according to the manufacturer's instructions.

The QC material, PreciControl ISD (PC ISD, Roche Diagnostics), is available at 3 concentrations. Value assignment for the PC ISD material is performed in an initial measurement of a newly produced accuracy control on the cobas e 601 system by the manufacturer. The analyte content determined in this manner is the "first-assigned value." This value is reported on the value sheet for the dedicated accuracy control lot. Thereafter, for the accuracy control lots that are shipped to individual sites for use, the accuracy control is measured on every instrument platform and in the current reagent standardization to determine the platform-specific target value. The QC samples were prepared and stored with the same method as 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 to specified ranges before patient sample material was measured.

In addition, each laboratory in this evaluation has a routine internal QC program. The laboratory in Klinikum-Stuttgart also used the QC material ClinCheck Control for immunosuppressants (RECIPE, levels 1, 2, and 3) and More Diagnostics Tac/CsA controls (levels 1, 2, and 3) for Dimension. For the proficiency testing samples in Stuttgart at Marienhospital, imprecision and inaccuracy of CMIA were evaluated with Bio-Rad Lyphochek Whole Blood Immunosuppressant Control (levels 1, 2, and 3). The laboratory in Munich used the Chromsystems MassCheck controls (levels 1, 2, 3, and 4). The laboratory in Barcelona used 3-level controls containing CsA in blood lysate (ClinChek Whole Blood Control for Immunosuppressants, lyophilized, levels 1, 2, and 3; RECIPE). The laboratory in Ghent used Chromsystems MassCheck Immunosuppressants Whole Blood control, levels 1 and 3, for LC-MS/MS; Bio-Rad Abbott IS-MCC controls for the Architect; and More Diagnostics Tac/CsA controls for Dimension.

During the 7-month evaluation period, adverse event reports for the CMIA assay were posted on the FDA website indicating arbitrary values for sample recovery related to certain reagent lots. The sites in Ghent and Brussels were affected by the sample recovery issue. The site in Ghent was able to continue experiments with a different CMIA lot that was not affected. Brussels had access only to the affected lots; therefore, the method comparison experiments in Brussels were discontinued.

LC-MS/MS Measurements

Each investigational site performed tandem mass spectrometry measurements according to protocols developed and routinely used at the site. Table 1 outlines the LC-MS/MS method details used by each laboratory.

Sample Sources and Handling

EDTA whole blood samples from patients who had received an organ transplant and were under CsA therapy were collected at the respective sites (except Ghent) for

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TABLE 1. Details of LC-MS/MS Methods Used by Investigational Sites in the MCE

Laboratory	Instrument (HPLC/MS)	Working Range, mcg/L	Sample Preparation	Internal Standard	Calibration (Daily)	Chromatographic Separation (Analytical Column; Mobile Phase; Column Temperature; Injection Volume; Run Time)
Barcelona	Waters Acquity UPLC/ Waters TQD	15–1963	Protein precipitation with $ZnSO_4$	[D12]-Cyclosporine A	Seven-level calibrators (ClinCal whole blood calibrator set, lyophil., level 0–6; RECIPE)	MassTrak TDM C18 2.1 × 10 mm (Waters); A: 0.1% (vol/vol) formic acid and 2 mmol/L ammonium acetate in water, B: 0.1% (vol/vol) formic acid and 2 mmol/L ammonium acetate in methanol; 55°C; 20 μL in 50 μL loop; 2.5 min run time
Brussels	Agilent 1290 Infinity/ Agilent 6460 series	20–1250	Protein precipitation with $ZnSO_4$	[D12]-Cyclosporine A	"Home made" with RECIPE calibrators	Zorbax Eclipse XDB C18 4.6 × 50 mm (Agilent); A: 60% methanol (aq), B: 2 mmol/L ammonium acetate, 0.5% formic acid (aq), 5% acetonitrile with 95% methanol; 60°C; 40 μL; 3 min run time
Ghent	Waters Acquity UPLC/ Waters TQD	4.76–1250	Protein precipitation with $ZnSO_4$	[D12]-Cyclosporine A	Chromsystems MassCheck Immunosuppressants whole blood calibrator (one point, in duplicate) (Chromsystems)	MassTrak TDM C18 2.1 × 10 mm (Waters); A: 2 mmol/L ammonium acetate and 0.1% formic acid in water; B: 2 mmol/L ammonium acetate and 0.1% formic acid in methanol; 50°C; 10 μL; 1.8 min run time
Munich	Waters Alliance 2795/ Waters Quattro Ultima Pt	43–913	Protein precipitation with $ZnSO_4$ followed by on-line solid phase extraction	4-fold deuterated cyclosporine A	6-point; Chromsystems Calibrator Set (Chromsystems)	Sunfire C18 5 μm, 2.1 × 100 mm (Waters); 90% methanol/10% 2 mmol/L ammonium acetate; 50°C; 10 μL; 4.5 min run time
Stuttgart[16,17]	Waters Alliance 2695/ Waters Quattro micro API	10–1800	Protein precipitation with $ZnSO_4$	Cyclosporine D	6-point; Chromsystems Calibrator Set (Chromsystems)	MZ Aqua Perfect C18 5 μm, 150 × 3.0 mm (MZ-Analysentechnik); A: 2 mmol/L ammonium acetate, 0.1% formic acid (aqueous), B: 2 mmol/L ammonium acetate, 0.1% formic acid in methanol; 65°C; 30 μL; 5.5 min run time

the imprecision, LLOQ, linearity, and method comparison experiments. Not all transplant types were available at all investigational sites; therefore, additional residual samples from all transplant cohorts (N = 70 per cohort) were supplied by Roche Diagnostics to the sites in Stuttgart, Barcelona, Brussels, and Ghent. These additional samples were collected at the Institute for Clinical Chemistry, Medical University of Hannover under the same Institutional Review Board/Ethics Committees and Good Clinical Practice guidelines as outlined above. Commercial proficiency testing samples were purchased from Analytical Services International Ltd (London, United Kingdom). Additional patient samples with CsA at high concentration were purchased from a commercial laboratory (Cerba Specimen Services, France). The transplant cohorts of these samples were not identifiable and are indicated as "unknown" in the results. The number of samples measured for each experiment is described in the Results section.

Samples were prepared for immunoassay measurement as described above. Samples not collected at site were shipped frozen on dry ice in a thermal container and inspected upon arrival to ensure sample integrity, and stored at −15°C

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or below until testing occurred. Samples collected at site were stored at room temperature (18–25°C) if tested within 8 hours of collection or at 2–8°C if tested within 1 week. If longer storage was necessary, samples were stored below −15°C. Liquid whole blood aliquots used for the method comparison were measured within 24 hours using all methods. Between method comparison measurements, the aliquots were stored at 2 to 8°C. Aliquoted samples were treated in a defined way and did not undergo more than 1 freeze/thaw cycle. Frozen samples were thawed and mixed thoroughly before use. Whole blood samples were never centrifuged.

Imprecision and Inaccuracy

Assay imprecision was tested in Stuttgart and Ghent according to protocol CLSI EP5-A2[18] on a cobas e 411 system and a cobas e 602 system. Imprecision was determined using 84 aliquots each of the PreciControl ISD material at 3 concentrations and of 5 pooled EDTA whole blood samples with defined concentration ranges from 30 to 2000 mcg/L (Table 2). Controls and samples were frozen in 300 μL aliquots and stored below −15°C. For each measuring day, 1 aliquot of each sample and control was thawed. Before the first run on day 1, instrument calibration was performed. One run was performed per day over 21 days, and samples were randomized. Four replicates per sample were measured. Mean, median, minimum, maximum, within-run, intermediate imprecision [SD and coefficient of variation (CV)%], and inaccuracy were calculated in the Windows-based Computer Aided Evaluation (WinCAEv) program (Roche Diagnostics[19]). Acceptance criteria for intermediate imprecision were based on Oellerich et al[20]: at a concentration of 50 mcg/L, CV ≤10%; at a concentration of 300 mcg/L, CV ≤5%. Inaccuracy was calculated using the measurement results from the PreciControl ISD material.

Lower Limit of Quantification

LLOQ was assessed in Stuttgart and in Ghent on a cobas e 411 system and a cobas e 602 instrument.[21] Pooled EDTA whole blood samples with target concentrations at approximately 10, 20, 30, 40, and 50 mcg/L were used and 14 aliquots with a volume greater than 300 μL were prepared. Samples were measured in 1 run each day for 10 days in total with a single measurement per aliquot. Mean, median, minimum and maximum values, SD, and CV were calculated in WinCAEv.

Linearity

Linearity testing was performed in Stuttgart and Ghent on a cobas e 411 system and a cobas e 602 system according to the polynomial method described in CLSI EP6-A[22] that evaluates nonlinearity. The method consists of 2 steps. The first step examines whether a nonlinear polynomial equation provides a superior fit to the data than a linear equation. The second step assesses whether the differences between the best-fitting nonlinear and linear equation are less than the specified bias for ECLIA. As the medically relevant range for trough sampling covers mainly the lower part of the measuring range, weighting functions (quadratic and inverse concentration) were applied to test results of the dilution series. The dilution series were prepared as follows: A 24 mL pool of whole blood sample with low CsA concentration was prepared for the experiment. A 12-mL aliquot of this pool was spiked with CsA to a concentration greater than 2100 mcg/L. The

TABLE 2. Imprecision and Bias of the ECLIA Assay

	Target Concentration, mcg/L	Within-Run (CV), %	Intermediate (CV), %	Mean, mcg/L	Bias (Mean), %
Stuttgart cobas e 411					
PreciControl ISD level 1	66.0	4.2	6.8	72	10
PreciControl ISD level 2	324	3.4	4.7	345	7
PreciControl ISD level 3	1202	3.2	4.6	1329	11
HSP 1: 30–90 mcg/L		3.8	8.5	53	
HSP 2: 90–300 mcg/L		2.8	5.0	143	
HSP 3: 300–600 mcg/L		3.5	4.7	468	
HSP 4: 700–1400 mcg/L		3.1	5.3	1154	
HSP 5: 1500–2000 mcg/L		4.2	5.8	1880	
Ghent cobas e 602					
PreciControl ISD level 1	72.7	4.6	6.1	76	4
PreciControl ISD level 2	336	3.3	4.9	334	0
PreciControl ISD level 3	1282	3.1	4.7	1251	−2
HSP 1: 30–90 mcg/L		4.5	6.6	63	
HSP 2: 90–300 mcg/L		3.7	5.0	146	
HSP 3: 300–600 mcg/L		2.6	4.2	378	
HSP 4: 700–1400 mcg/L		3.7	5.1	1026	
HSP 5: 1500–2000 mcg/L		3.0	5.4	1769	

Results from measurements in Stuttgart and Ghent are shown. Within-Run and Intermediate CVs are expressed as the one-sided 95% confidence interval (upper confidence limit). Approximate confidence limits for the coefficient of variation are calculated by normalization of the standard deviation to the respective mean.

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high concentration sample pool was then diluted with the low concentration sample pool to create a dilution series of 15 steps. In addition, a patient sample high concentration pool was used to create a dilution series with a low concentration pool. Three aliquots per dilution step were prepared and measured in 1 run as 3 replicates per dilution step. Linearity was assessed using absolute deviation from the calculated dilution concentration in Microsoft Excel (Microsoft, Redmond, WA). The polynomial regression analysis for first-, second-, and third-order polynomials and the mean, median, minimum and maximum values, SD, and CV were calculated in WinCAEv.

External Quality Control Scheme

External QC samples from the Ciclosporin IPTS (ASI Ltd, London, United Kingdom) were measured at all investigational sites. The scheme includes samples that are either spiked to a known concentration ("spiked" samples) or pooled samples from patients receiving CsA immunosuppressive therapy ("pooled patient" samples). IPTS samples were measured with ECLIA on 3 different cobas platforms, with LC-MS/MS, and with CMIA and ACMIA at selected sites. In this evaluation, both spiked and pooled patient samples were measured. Details of sample measurement per site and assay method are outlined in the supplemental data (see **Table 1, Supplemental Digital Content 1**, http://links.lww.com/TDM/A84). All instruments were calibrated before the experimental run. Samples were divided into 300 μL aliquots and measured in 1 run with 3 replicates for each sample. Mean, median, and percent recoveries were calculated in WinCAEv.

Method Comparison

The method comparison experiment was performed at all investigational sites between the ECLIA on cobas e 411, cobas e 601, and cobas e 602 platforms and LC-MS/MS instruments; between ECLIA and CMIA in Stuttgart, Ghent, and Brussels; and between ECLIA and ACMIA in Stuttgart and Ghent. Due to adverse event-related recovery problems with certain CMIA assay lots, the method comparison experiments in Brussels with this platform were discontinued. Residual samples from patient cohorts for liver, kidney, heart, lung, and bone marrow transplant were measured with 1 replicate per method. More than 60 samples per transplant type were measured, with the total number of samples measured indicated in the respective plots in the Results section. Slope, intercept, and Pearson's r were calculated in WinCAEv. Discrepant results were handled as outlined below. Comparisons were calculated using weighted Deming regression. The intermediate imprecisions for each LC-MS/MS method from the investigational sites were used to calculate the imprecision ratio in the weighted Deming regression. Acceptance criteria against LC-MS/MS were based on the recommendation of Oellerich et al[20] and defined as a slope of 1.00 ± 0.10 and an intercept of ±15 mcg/L or less.

Discrepant Results

Discrepant results were compared with the respective result obtained with tandem mass spectrometry. All samples showing more than 40% difference to LC-MS/MS results were regarded as discrepant. If sufficient sample volume remained, the discrepant sample was retested in triplicate using all assays. If insufficient sample volume remained for retesting with all assays, testing was repeated in 1 of 2 ways. Either the sample was remeasured in triplicate using the assay(s) which showed the most discrepant value as compared with the other assays used at that site, or the sample was remeasured in single or duplicate for 2 or more assays used at that site, according to the remaining sample volume available.

RESULTS

Imprecision and Inaccuracy

Results for within-run and intermediate imprecision of the ECLIA assay are given in Table 2. In the human sample pools at concentrations less than 90 mcg/L, the within-run CV was below 4.5% at both sites. At a concentration between 90 and 600 mcg/L, the CV values were 3.7% or less for both sites. At concentrations ≥700 mcg/L, CVs ranged from 3.0% to 4.2%. Intermediate imprecision results in Stuttgart were a CV of 8.5% and in Ghent 6.6% for the sample pool with a target concentration less than 90 mcg/L (acceptance criterion ≤10%). Between 90 and 600 mcg/L, the CV's for both sites were 5.0% or less (acceptance criterion ≤5%). At concentrations ≥700 mcg/L, CVs ranged from 5.1% to 5.8%. Bias as evaluated with the PreciControl ISD material (Table 2) ranged from −2% to 11%.

Lower Limit of Quantification

The LLOQ of the assay was determined as 6.8 mcg/L in Stuttgart and 1.8 mcg/L in Ghent at 20% CV. The concentrations of 29.7 mcg/L in Stuttgart and 15.9 mcg/L in Ghent could be determined with a CV of 10% (Fig. 2).

Linearity

Figure 3 shows the results from Stuttgart and Ghent for the linearity experiment, with both the patient pool and spiked sample pools plotted as the expected concentration against the absolute deviation of each sample from the expected result as calculated by the linear model. The maximum difference

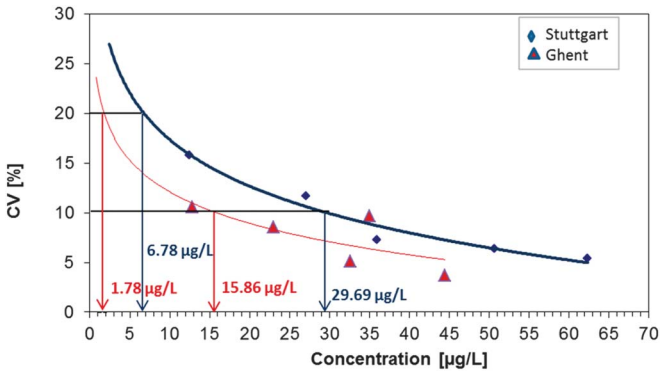


FIGURE 2. Lower limit of quantification of the ECLIA assay. LLOQ was assessed via measurement of 5 samples at low CsA concentration at 2 investigational sites on 2 different cobas platforms. CsA recovery at 10% and 20% CV are indicated for each site.

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between linear and quadratic models was 11.9%, and the maximum difference between linear and cubic models was 8.9%.

External Quality Control Scheme

Results from external QC (IPTS) sample measurements obtained in this MCE are outlined in Table 3, as well as overall results from the participating IPTS laboratories as summarized in the program reports. The mean and SD of the measurements from all sites per method are indicated, and comparison of each method to the IPTS reported overall "HPLC/MS" results (which includes both HPLC/MS and HPLC/MS/MS methods) are reported separately for pooled patient and spiked samples.

Comparison of the mean results with pooled patient samples from the MCE measurements between ECLIA and the "HPLC/MS" overall results from IPTS shows a difference of 11%, while it is 4% and 19% for ACMIA and CMIA MCE results and 2% and 5% for the overall ACMIA and CMIA results. Comparison of the spiked samples on average yielded smaller differences. Comparing ECLIA mean MCE results with spiked samples to "HPLC/MS" overall results yields a difference of 4%, −5%, and 12% for ECLIA, ACMIA and CMIA results, respectively. For the overall results, the differences are 2% for ACMIA and MCIA. The External Quality Assessment Schemes sample recovery distributions per instrument for each sample are also displayed using box-and-whiskers plots (see **Figure 1, Supplemental Digital Content 1**, http://links.lww.com/TDM/A84).

Method Comparison

Method comparison by weighted Deming regression analysis and Bland–Altman graphical representation of the values obtained from each method are displayed in Figures 4A–F. Figure 4A is the Deming regression of the LC-MS/MS measurements from all sites versus ECLIA, with 1643 data points. The slope is 1.04 [95% confidence interval (CI), 1.03–1.06; acceptance criterion: 0.9–1.1], the intercept is 2.8 mcg/L (95% CI, 1.5–4.1 mcg/L; acceptance criterion: ±15 mcg/L or less), and the Pearson's r is 0.98. All values are within the acceptance criteria outlined by Oellerich et al.[20] Figure 4B shows a Bland–Altman difference plot of the LC-MS/MS comparison data ≤500 mcg/L, with the normalized difference expressed as percentage on the y axis. The mean bias is 7.5% of the average, with 2 SD limits of −21.1% and 36.2%. Samples from the heart transplant cohort that had results outside of the 2 SD limits showed a trend toward deviation greater than the upper 2 SD limit at concentrations greater than 200 mcg/L, and bone marrow samples outside of the 2 SD limit trended toward deviation less than the lower 2 SD limit.

Results of the comparison between ECLIA and CMIA are shown in Figures 4C and D. As described in the Materials and Methods, some CMIA lots had adverse event reports posted on the FDA Web site during the course of this study with arbitrary values affecting CsA recovery. The sites in Ghent and Brussels also experienced an effect on analyte recovery due to these technical issues. The site in Ghent was able to continue measurements with an unaffected lot already on hand. The Deming regression analysis in Figure 4C using 682 paired data points yielded an equation of $y = 1.40 + 0.87 \times$ (95% CI slope, 0.85–0.89; 95% CI intercept, −0.89 to 3.7 mcg/L), with a Pearson's r of 0.98. While the Pearson's r is close to 1, the slope shows a greater difference between ECLIA and CMIA than between ECLIA and LC-MS/MS methods (see also **Figure 2A, Supplemental Digital Content 1**, http://links.lww.com/TDM/A84, comparison of LC-MS/MS and CMIA). The Bland–Altman plot (Fig. 4D) of the same data set also reflects this bias by CMIA in comparison to ECLIA results for most transplant types, with a mean bias of −12.2% and 2 SD limits of 19.8% and −44.1%. The heart samples showed a trend to be greater than the upper 2 SD limit of 19.8%.

Comparison of ECLIA to ACMIA is displayed in Figures 4E and F, with 679 paired data points used in the regression analysis. The slope determined is 0.96 (95% CI, 0.93–0.98), the intercept is −4.15 mcg/L (95% CI, −7.1 to −1.2 mcg/L), and the Pearson's r is 0.98. These results are comparable to the comparison of ECLIA with LC-MS/MS

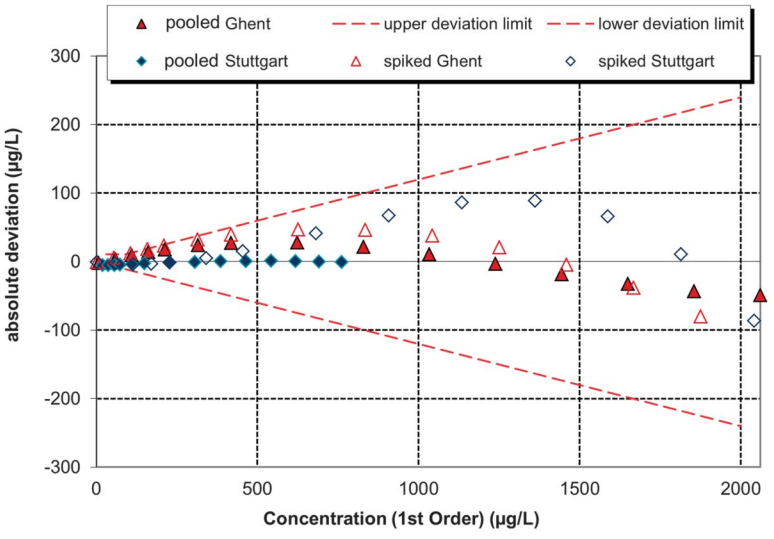


FIGURE 3. Linearity assessment of the ECLIA assay in pooled patient blood and spiked samples. Linearity was assessed according to CLSI EP6-A using 15 dilution steps at 2 investigational sites on 2 different cobas platforms with a pooled patient and a spiked sample pool. The data were fitted to a linearity model. The absolute deviation to the model for each dilution point is indicated on the y axis.

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TABLE 3. External Quality Control Scheme Results From IPTS Samples (Both MCE Data and Overall External Quality Control Data From IPTS Scheme)

	328	329			335	337	
IPTS Sample Number	**A Spiked**	**A Spiked**	**B Pooled**	**C Spiked**	**B Spiked**	**A Spiked**	**B Spiked**
ECLIA MCE, N	12	12	12	12	15	15	15
Mean ± SD, mcg/L	494.0 ± 25.7	26.8 ± 2.8	205.5 ± 6.4	367.9 ± 15.4	541.7 ± 14.1	55.1 ± 4.9	160.9 ± 6.3
LC/MS–MS MCE, N	9	9	9	9	15	15	15
Mean ± SD, mcg/L	508.0 ± 30.4	27.6 ± 2.7	184.2 ± 16.8	371.9 ± 20.3	502.3 ± 54.1	55 ± 3.6	149.1 ± 7.4
HPLC/MS overall, N	118	117	124	124	123	133	133
Mean ± SD, mcg/L	487.0 ± 28.5	27.4 ± 4.2	186.5 ± 13.4	357.8 ± 26.7	506.7 ± 36.9	53.8 ± 4.4	154.2 ± 11.8
ACMIA MCE, N	6	6	6	6	6	6	6
Mean ± SD, mcg/L	475.7 ± 12.0	22.5 ± 2.5	205.1 ± 22.3	386.2 ± 49.5	513.2 ± 13.1	48.2 ± 4.5	157.0 ± 8.2
ACMIA overall, N	138	78	88	88	87	89	89
Mean ± SD, mcg/L	500.3 ± 39.0	27.4 ± 6.6	194.6 ± 14.1	390.0 ± 22.9	530.7 ± 45.1	55.2 ± 7.2	162.6 ± 9.6
CMIA MCE, N	6	6	6	6	9	9	9
Mean ± SD, mcg/L	479.1 ± 61.1	29.6 ± 4.2	209.3 ± 9.1	386.0 ± 20.3	567.4 ± 36.5	52.4 ± 11.0	155.4 ± 19.6
CMIA overall, N	133	91	107	106	111	110	110
Mean ± SD, mcg/L	517.8 ± 54.4	28.1 ± 4.2	194.8 ± 24.4	358.5 ± 47.7	527.8 ± 53.7	56.2 ± 8.7	153.7 ± 17.6

	338	339	340	342
IPTS Sample Number	**A Pooled**	**C Pooled**	**C Spiked**	**A Spiked**
ECLIA MCE, N	15	6	15	6
Mean ± SD, mcg/L	134.1 ± 5.8	190.1 ± 4.1	118.8 ± 6.3	478.4 ± 14.4
LC/MS–MS MCE, N	15	6	15	6
Mean ± SD, mcg/L	117.6 ± 8.9	171.1 ± 8.8	116.0 ± 14.4	444.3 ± 28.1
HPLC/MS overall, N	132	135	134	140
Mean ± SD, mcg/L	120.4 ± 8.1	170.0 ± 13.6	115.5 ± 8.6	444.4 ± 37.2
ACMIA MCE, N	6		6	
Mean ± SD, mcg/L	117.1 ± 5.5		102.4 ± 2.7	
ACMIA overall, N	89	87	84	83
Mean ± SD, mcg/L	119.4 ± 9.8	176.2 ± 13.3	107.4 ± 10.4	453.4 ± 27.1
CMIA MCE, N	9	3	9	3
Mean ± SD, mcg/L	142.2 ± 16.7	216.2 ± 29.5	124.5 ± 19.5	652.8 ± 19.5
CMIA overall, N	105	100	109	111
Mean ± SD, mcg/L	126.2 ± 17.3	181.5 ± 20.3	116.0 ± 15.1	464.9 ± 52.5

Values shown are the mean of a given method's measurement results from all participating laboratories, including replicates. Results from the current MCE Study are indicated by "MCE". "Pooled" is pooled patient blood samples. "N" is number of samples.

but also show a bias by ACMIA in comparison to the ECLIA results (see also **Figure 2B, Supplemental Digital Content 1**, http://links.lww.com/TDM/A84). Bland–Altman analysis (Fig. 4F) confirms this observation with a mean bias of −8.8% between the 2 results over the entire measuring range, and upper and lower 2 SD limits of 20.7% and −38.3%, respectively. By visual inspection, the transplant types were distributed equally over the 2 SD limits.

These method comparison data are also displayed as Deming regression analysis, with each site indicated by a different symbol in the **Supplemental Digital Content 1** (see **Figure 3A–C**, http://links.lww.com/TDM/A84). In addition, the individual Deming regression for comparison between ECLIA and LC-MS/MS for each transplant type is shown in the **Supplemental Digital Content 1** (see **Figure 4A–E**, http://links.lww.com/TDM/A84). Discrepant results were observed for 81 samples (3%). After re-analysis, the discrepancy was resolved in 72 cases and remained in 9 cases.

With respect to comparison of sample throughput, ECLIA as well as CMIA and ACMIA can process approximately 70 samples per hour, whereas the LC-MS/MS method allows a throughput of roughly 10–30 tests per hour, depending on the specific method parameters and run time.

DISCUSSION

In an MCE of the new ECLIA assay conducted at 5 study sites, good assay imprecision, with linearity over a wide measuring range and close agreement with LC-MS/MS methods, and proficiency testing results were observed. The LLOQ of the test (at an interassay coefficient of variation of <20%) was found to be less than 10 mcg/L. This is substantially below the target range of CsA. Within the range of the typical therapeutic window, CVs clearly below 10% were observed. According to the linearity experiment results, the ECLIA assay is linear in the range of 30–2000 mcg/L. The absolute deviation from a linear fit to the results increases toward

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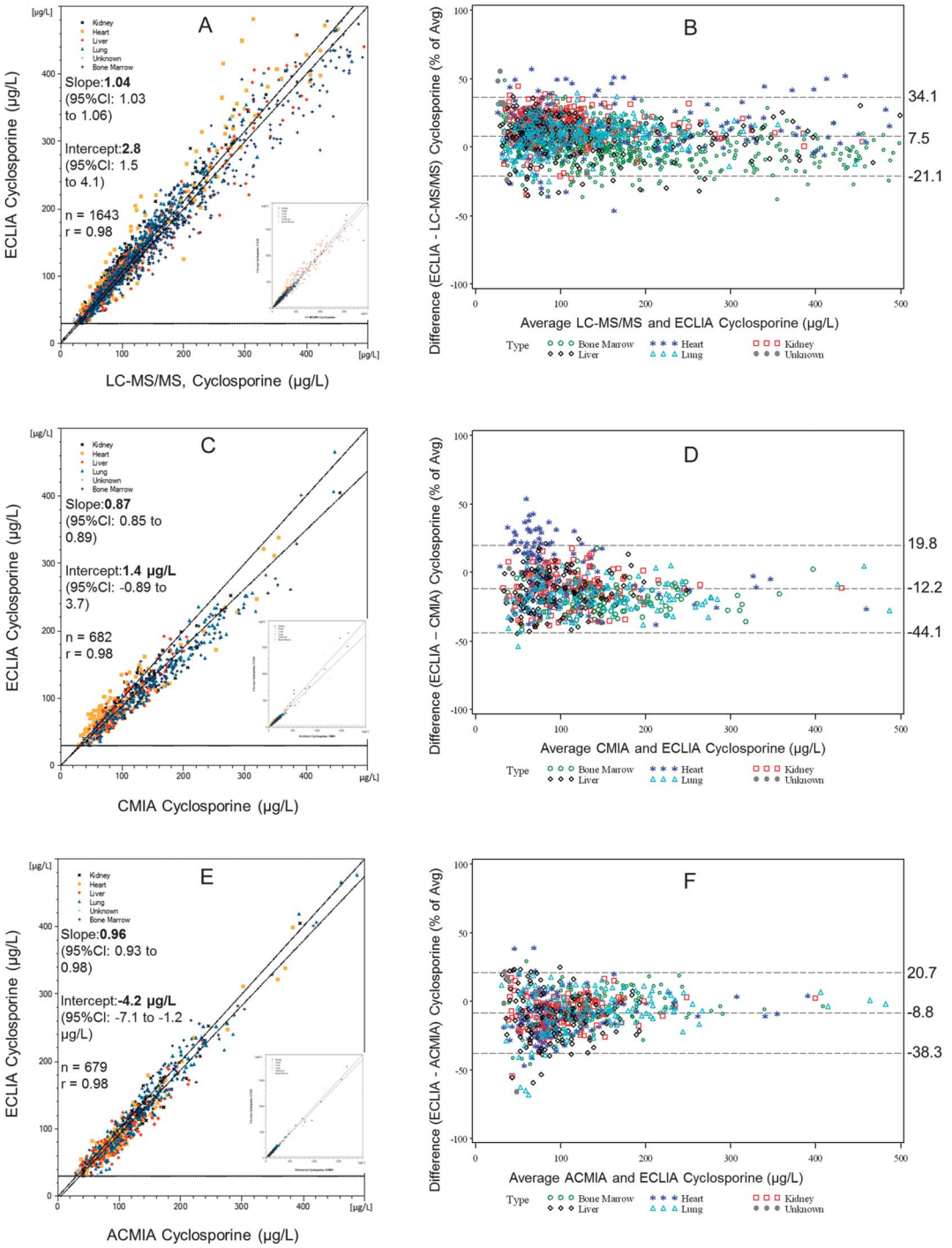


FIGURE 4. A–F, Method comparison of the ECLIA assay versus LC-MS/MS, CMIA, and ACMIA. Method comparison of the ECLIA assay against LC-MS/MS (A, B), CMIA (C, D), and ACMIA (E, F) was performed using both weighted Deming regression and Bland–Altman analysis. Data points from 0 to 500 mcg/L are displayed in the full-sized figure and the full range of the data points is displayed as an inset in the graph. Regression parameters and 95% CI (calculated using the entire measuring range) are indicated within the plot. Five transplant cohorts and commercially obtained samples of unknown transplant type are indicated with different symbols in each graph. The dashed line in the regression plots parallel to the *x* axis represents the lower limit of the ECLIA assay measuring range, the unity line, and the fitted line. Bland–Altman plots with data from 0 to 500 mcg/L are displayed using the average of the 2 methods on the *x* axis and the normalized difference in percentage on the *y* axis. The bias and ±2 SD range are indicated with dashed lines.

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the higher end of the measuring range, as expected based on the weighting function applied to the dilution series results.

The recommendations for intermediate imprecision according to Oellerich et al[20] were met for samples <700 mcg/L. Applying the same criterion (CV ≤5%) for all concentrations ≥300 mcg/L, the criterion was not met for 2 samples, human sample pools 4 and 5, in both Stuttgart and Ghent. The highest observed CV in this range was 5.8% in Stuttgart. It must be noted, however, that for the most widely used CsA immunoassay, the CMIA method, CVs ranging from 6.6% to 14.3% in the concentration range from 343 to 464 mcg/L are reported in a multicenter study.[23] Thus, the data from the ECLIA evaluation demonstrate a superior assay imprecision.

The analysis of a representative set of proficiency testing samples demonstrated good agreement of ECLIA results with the average results of overall LC-MS/MS user laboratories who participated in the external quality scheme, with a difference of 11% for pooled patient IPTS samples and 4% for spiked samples. It is worthwhile noting that pooled patient samples may have the result that extremes of CsA metabolism are averaged out, preventing a true "real-world" picture of cross-reactivity ranges in a set of individual patient samples. Nonetheless, the observation that ECLIA results from IPTS pooled patient samples gives results that are 11% higher than LC-MS/MS, and 4% higher for spiked samples, suggests that the ECLIA calibrators are not biased.

These observations are in line with the comparative measurement of more than 1600 samples using 5 different LC-MS/MS methods in this multicenter study: close correlation (Pearson's $r = 0.98$) was found with a slope of 1.04 (95% CI, 1.03–1.06) and a negligible intercept. Assessment of the paired results (1516 data points at ≤500 mcg/L) according to Bland and Altman also demonstrated slightly higher results, with ECLIA displaying a mean difference of +7.5% compared to LC-MS/MS. Cross-reactivity with metabolites commonly found in whole blood samples for the ECLIA assay was evaluated during assay development by the manufacturer, based on guidance from CLSI document EP7-A2. At 2000 mcg/L, cross-reactivity was 6% for AM9, 2% for AM1 and AM4n, and not detectable for AM1c, AM1c9, and AM19 (see **Table 2, Supplemental Digital Content 1**, http://links.lww.com/TDM/A84).[24] Consequently, this small bias is most likely explained by some degree of between-method variance in LC-MS/MS standardization rather than the ECLIA assay calibrators, as mentioned above. However, the standardization of the test is in full agreement with the recommendations of a consensus panel on CsA monitoring.[20] In the article from the consensus panel, a slope of ≤10% when compared with a chromatographic method as a reference is recommended to demonstrate that a method accurately measures CsA concentration.

When compared to LC-MS/MS, the CMIA assay had a substantially higher slope (1.28) for a set of 697 samples (see **Figure 2A, Supplemental Digital Content 1**, http://links.lww.com/TDM/A84). This is surprising, as results from an MCE of the CMIA assay reported slopes of 0.87–0.92 in comparison to LC-MS/MS.[23] In addition, measurement of the proficiency testing samples demonstrated good agreement between CMIA results and LC-MS/MS methods. However, the original CMIA measurements reflected in the overall proficiency testing results were performed at an earlier time point, most likely with different reagent lots than those used in the MCE. This difference is a plausible cause for the different slopes observed in the method comparisons.

Test handling of the ECLIA assay was found to be convenient, although it involves a manual protein precipitation step preceding the automated analysis. This step accounts for additional workload compared to usual immunoassay tests; however, protein precipitation is expected to address the issue of heterophilic antibodies. Such anti-reagent antibodies represent a major pitfall of immunoassays, and removal of antibodies consequently contributes to improved patient safety.[25,26] Nevertheless, this precipitation step requires labeling and handling of one additional tube and must be recognized as a potential source of gross error when compared to other fully automated assays implemented on cobas analyzers.

The assay offers a wide measuring range up to 2000 mcg/L. This is of relevance regarding postapplication sampling strategies, as done in AUC studies and mainly in the C2-approach, with sampling 2 hours after oral administration of CsA.[27,28] Although this approach is rarely applied in the countries represented by the investigational sites in this evaluation, it is an important option for the test to be applicable in this setting without need for sample dilution.

The concept of an MCE of a new commercial reagent tries to address as many recognized and unrecognized variables of different instruments and sites as possible (different instrument lots; differential pattern of other tests run on respective instrument, throughput, environmental conditions; skill of operators; shipment and storage of reagents, etc.) in an industry–academic cooperation. In the particular case of CsA, comparative measurement with different systems in use at the different sites and heterogeneous LC-MS/MS methods were of particular importance. Consequently, this extensive set of data allows for a thorough appraisal of the test performance before it is commercially available. Nevertheless, long-term stability and lot-to-lot consistency of an immunoassay is an essential challenge for assay manufacturers, requiring careful ongoing assessment of the assay performance, both by the manufacturer and by the users.

CONCLUSION

In summary, we conclude from the data of this large MCE that the new ECLIA assay is fit for its purpose, the therapeutic drug monitoring of CsA in transplantation medicine.

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