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Research Article

Automated LC-MS/MS: Ready for the clinical routine Laboratory?

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ABSTRACT

Background: Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a sensitive method with high specificity. However, its routine use in the clinical laboratory is hampered by its high complexity and lack of automation. Studies demonstrate excellent analytical performance using the first fully automated LC-MS/MS for 25-hydroxy vitamin D and immunosuppressant drugs (ISD) in hospital routine laboratories.
Objectives: Our objectives were (1) to verify the suitability of an automated LC-MS/MS in a commercial laboratory, which differs from the needs of hospital laboratories, and (2) examine its usability among operators with various professional backgrounds.
Methods: We assessed the analytical assay performance for vitamin D and the ISDs cyclosporine A and tacrolimus over five months. The assays were compared to an identical analyzer in a hospital laboratory, to in-house LC-MS/MS methods, and to chemiluminescent microparticle immunoassays (CMIA). Nine operators evaluated the usability of the fully automated LC-MS/MS system by means of a structured questionnaire.
Results: The automated system exhibited a high precision (CV < 8%), accuracy (bias < 7%) and good agreement with concentrations of external quality assessment (EQA) samples. Comparable results were obtained with an identical analyzer in a hospital routine laboratory. Acceptable median deviations of results versus an in-house LC-MS/MS were observed for 25-OH vitamin D3 (-10.6%), cyclosporine A (-4.3%) and tacrolimus (-6.6%). The median bias between the automated system and immunoassays was only acceptable for 25-OH vitamin D3 (6.6%). All users stated that they had had a good experience with the fully automated LC-MS/MS system.
Conclusions: A fully automated LC-MS/MS can be easily integrated for routine diagnostics in a commercial laboratory.

1. Introduction

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is considered the gold standard for many areas of clinical chemical diagnostics. Despite the advantages of LC-MS/MS over immunoassays in terms of analytical specificity, sensitivity, and multiplexing [1–3], immunoassays are still employed by over 90% of participants registered in the Birmingham Quality UK National External Quality Assessment Scheme (UK NEQAS) scheme for vitamin D (Round 153, April 2022) and about half of the participants in the LGC Immunosuppressant Proficiency Testing (IPT) scheme (Round 456, April 2022) [4,5].

Hurdles for a broader use of LC-MS/MS in routine laboratories are

Abbreviations: BSA, bovine serum albumin; CI, Confidence interval; CMIA, Chemiluminescent Microparticle Immunoassay; CLSI, Clinical & Laboratory Standards Institute; CRM, Cerilliant-Certified Reference Material; CV, coefficient of variation; ECLIA, Electrochemiluminescence Immunoassay; EDTA, Ethylenediaminetetraacetic acid; ERM, European Reference Material; EQA, External Quality assessment; IPT, Immunosuppressant Proficiency Testing; ISD, Immunosuppressant Drugs; IVD, In Vitro Diagnostic; JCTLM, Joint Committee for Traceability in Laboratory Medicine; LC-MS/MS, Liquid Chromatography-tandem Mass Spectrometry; LDT, laboratory-developed test; LLOQ, Lower Limit of Quantification; NaCl, sodium chloride; NAT, nucleic acid testing; NEQAS, National External Quality Assessment Scheme; NIST, National Institute of Standards and Technology; R, Pearson correlation coefficient; R_s, Spearmańs rank correlation coefficient; SD, Standard Deviation; SRM, Standard Reference Material.

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high initial costs of equipment, the requirement for personnel with a high level of technical expertise, complex method validation, and lack of automation [6]. Additionally, assays for LC-MS/MS are typically laboratory-developed and often involve manual and time-consuming sample and reagent preparation, as well as a high diversity and variable quality of instrumental equipment leading to a lower degree of result harmonization between laboratories [7,8]. An advantage of immunoassays is that they are typically integrated into automated devices and in vitro diagnostic (IVD)-certified for routine use [9]. Thus, they can be conveniently integrated into a laboratory workflow, performed easily and quickly around the clock, and mostly in a standardized manner which is less prone to technical errors. However, disadvantages of immunoassays include a lengthy development time for new assays for emerging analytes, lack of concordance between different lots of immunoreagents, and variable manifestation of non-specific binding and matrix phenomena (e.g. Hook effect, anti-reagent antibodies, heterophilic antibodies, cross-reactivity to structurally similar molecules) with different assay formats [9,10]. This classifies the immunoassay technology as inflexible for a quick reaction to new clinical requests and hampers between-assay consistence of results. Poor specificity can result in insufficient sensitivity and false results. Moreover, immunoassays are less suitable for high-throughput analysis compared to LC-MS/MS and sometimes still need manual sample pretreatment; this is the case with most whole blood immunosuppressant assays.

Over the past decade, efforts have been made to increase the level of automation in routine LC-MS/MS analysis in order to make this high-performance technology more accessible to non-specialist clinical laboratories. Liquid-handling platforms for automated sample preparation, along with validated assay kits, are now an option to reduce manual sample preparation steps, increase throughput, and harmonize results between laboratories [11,12]. More recently, fully automated sample preparation modules that can be directly connected to online coupled conventional LC-MS/MS systems have allowed for continuous sample processing [13]. The next milestone in the automation of LC-MS/MS was the all-in-one Cascadion™ SM Clinical Analyzer (Thermo Fisher Scientific, Vantaa, Finland) [14], which was launched in Europe in 2018 but was most recently withdrawn from the market due to potentially strategic reorientation by the manufacturer [15].

The device combines an automated sample reading and pretreatment system with a LC-MS/MS system, enabling the online transfer of results to a laboratory information system. Accompanying CE IVD-certified, pre-validated assay kits are available for measuring 25-hydroxy (OH)-vitamin D2 and D3 in serum or plasma (Cascadion™ SM 25-Hydroxy-Vitamin-D-Assay), and for simultaneous testing of the immunosuppressant drugs (ISDs) cyclosporine A, everolimus, sirolimus, and tacrolimus (Cascadion™ SM Immunosuppressants Panel) in whole blood.

Published results for both assay kits indicated good analytical performance of the fully automated LC-MS/MS analyzer and high levels of consistency between laboratories [16–22]. Therefore, it was suggested that such a device is ideally suited for high-quality 24/7 diagnostics in routine laboratories, particularly when specialized personnel are lacking. However, this high-level LC-MS/MS automation has only been tested in rather uniform hospital laboratory settings so far.

The aim of this study was to verify whether the performance and robustness of an all-in-one LC-MS/MS device meets the needs of commercial laboratories. To do so, we investigated the analytical performance of the Cascadion™ SM 25-OH vitamin D assay and the tests for cyclosporine A and tacrolimus within a large international laboratory network with more than 3,000 types of testing services and approximately 25,000 tests per day at the study site alone.

In addition, we compared the results with immunoassays for the Alinity platform (Abbott, Longford, Ireland), and with our in-house developed LC-MS/MS methods. Finally, a detailed survey of the user-friendliness of the analyzer was conducted among operators with different levels of expertise in the field of LC-MS/MS.

2. Materials and methods

2.1. Study design and blood sample collection

Leftover and anonymized blood and serum samples from SYNLAB laboratories in Leinfelden-Echterdingen, Augsburg, and Weiden, and from the clinical routine of the University Hospital of Tübingen were used for this study. Blood samples were collected between March 11, 2022 and August 04, 2022 in ethylenediaminetetraacetic acid (EDTA) tubes or serum tubes with clot activator. The samples were freshly analyzed on the day of reception with the respective local LC-MS/MS or immunoassay method directly from the primary sample tube, and the leftovers were transported to the Leinfelden laboratory. For comparison measurements, samples were then aliquoted into polystyrene push-cap tubes (Sarstedt, Nümbrecht, Germany) with a volume of at least 500 µl each. Aliquots were stored at 4 °C and analyzed within one week after sample entry. Before analysis, samples were mixed for 15 min on a roller-mixer.

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Commission of the State Chamber of Medicine in Baden-Württemberg (file number F-2022–12). As only fully anonymized patient samples were used that were not obtained specifically for use in this study through an interaction or intervention with living individuals, no informed consent was required.

2.2. The Cascadion™ SM clinical analyzer

The CE-IVD-certified Cascadion™ SM Clinical Analyzer (Thermo Fisher Scientific, Vantaa, Finland) is a fully automated LC-MS/MS analyzer with ready-to-use solvents, wash solutions, and assay reagents. The system consists of a liquid handling system equipped with a centrifuge, two parallel LC channels, and a triple quadrupole MS operated using a heated electrospray ionization source and selected reaction monitoring mode. Automated sample preparation for subsequent LC-MS/MS analysis includes protein precipitation followed by purification using a TurboFlow™ column (see Supplementary Table S1). The composition of the mobile phases and autosampler wash solvents is provided in Supplementary Table S2. The system was integrated into our laboratory information system for bidirectional transfer of test requests and results.

The Cascadion™ SM 25-OH Vitamin D Assay quantitates the concentrations of 25-OH vitamin D2 and D3 in serum or plasma samples separately within a single analytical run. The Cascadion™ SM Immunosuppressants Panel simultaneously quantitates cyclosporine A, everolimus, sirolimus, and tacrolimus from one whole blood (EDTA) sample aspiration. The manufacturer-created assay kits for 25-OH vitamin D and the immunosuppressants panel contain isotope-labeled internal standards (25-OH vitamin D2-d_3 and D3-d_6 and cyclosporine A-$^{13}C_2$-d_4, everolimus-d_4, sirolimus-d_3, and tacrolimus-^{13}C-d_2 respectively) in acetonitrile-ethanol solvent, calibrators (six levels), and internal quality controls (three levels) for analytic quantification. Stability and storage data of the kit components is provided in Supplementary Table S3.

Calibrators and on-board controls consist of a human serum matrix for the vitamin D assay or a human whole blood matrix for the ISD panel, spiked with known quantities of the analytes. According to the manufacturer, the vitamin D calibrators are traceable to the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) standard reference materials (NIST-SRM 972 set). The ISD panel assay calibrators are traceable to volumetrically prepared reference standards, which in turn are traceable to four recognized LC-MS/MS laboratories. The instrument requires monthly calibration, with each level analyzed in duplicate. Calibration concentrations are given in Supplementary Table S4.

The daily automatic startup procedure of the analyzer included initializing the robotic system, adjusting needles, rinsing pumps and needles, and checking the system to verify MS calibration validity. In

case the system check fails, MS calibration is automatically performed. Startup is completed by measuring startup blanks to equilibrate the instrument. All control levels were analyzed each day before patient samples were run, at intervals of every 30 samples, and after each calibration and column change. Only if the results of the control samples passed the acceptance criteria defined in the software (e.g., the Westgard rules [23] and peak quality), was measurement of patient samples possible.

Thanks to the synchronization of two LC-channels to a single mass spectrometer (runtime per channel: 5.2 min, of which the MS data collection window is approximately 1.5 min), results can be generated every 2.4 (vitamin D) to 2.6 (ISD) minutes. The instrument flags samples if chromatograms need to be visually checked. In case of minor errors concerning peak quality, the results must be accepted and commented manually (see Supplementary Fig. S1); in case of major errors, no result will be output. Manual integration or modification of the instrumental integration is not possible.

Further details of the fixed assay-related instrument parameters and the chromatographic and MS/MS rules implemented in the software are confidential and were not made available to us. The criteria used during data analysis are consistent with the Clinical and Laboratory Standards Institute (CLSI) C-62 guideline [24]. The robustness of the instrument was monitored by recording results and instrument errors over the course of the study period.

2.3. Analytical performance

Linearity was examined according to CLSI standard EP06-A using six dilutions of calibrator level 6 from the Cascadion™ SM assay kit and a blank sample [25]. ISD-negative pooled whole blood or a 0.9% solution of sodium chloride (NaCl, AppliChem, Darmstadt, Germany) supplemented with 7.5% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) for vitamin D served as diluents. Measurements were performed in duplicate (two aspirations per sample cup) on three days and results compared to theoretical concentrations. Linearity was accepted if deviation from linearity was within 15%, except for the lower limit of quantification (LLOQ) where 20% was allowed [26,27].

Potential carryover of 25-OH vitamin D or immunosuppressants was assessed over three days by analyzing two aliquots of the respective Cascadion™ SM calibrator with the highest concentration, followed immediately by a sample consisting of isotonic sodium chloride solution (0.9% NaCl, 7.5% BSA) or an ISD-negative blood sample, respectively.

Within-batch imprecision was assessed by consecutively running six aliquots of each of the three control levels with two aspirations per sample cup. Between-batch imprecision and bias were calculated from all daily quality control measurements between March 14, 2022, and August 12, 2022. In general, allowable levels for imprecision and bias are $< 15\%$ for chromatographic analytical methods [26,27], and a more stringent acceptance level of $< 10\%$ for both criteria was proposed for cyclosporine A and tacrolimus [28].

EQA samples were analyzed from the LGC (Treddington, UK) IPT scheme for cyclosporine A and tacrolimus and from the UK NEQAS scheme for vitamin D (Birmingham Quality, Birmingham, UK).

2.4. Comparative measurements

To assess inter-laboratory comparability patient samples were transported from the Leinfelden-Echterdingen laboratory to the Tübingen laboratory and vice versa at 4 °C and analyzed on Cascadion™ SM analyzers at both locations.

In addition, aliquots of patient samples containing vitamin D, cyclosporine A or tacrolimus were analyzed using CMIA immunoassays on the Abbott Alinity i immunoassay system (Abbott, Longford, Ireland) at the SYNLAB medical care centers in Leinfelden-Echterdingen (for vitamin D) and Augsburg (for cyclosporine A and tacrolimus). The immunoassays (Alinity i 25-OH vitamin D reagent kit, Alinity i cyclosporine reagent kit, Alinity i tacrolimus reagent kit) were performed according to the manufacturer's instructions and required several manual pre-treatment steps for whole blood samples (cyclosporine A and tacrolimus) [29–31]. Manufacturer's performance data for the immunoassays are given in Supplementary Table S5.

The Cascadion™ SM tests were compared to accredited in-house developed LC-MS/MS methods [32], which had been fully validated using a protocol consistent with IVD regulation requirements in the European Union (EU) [33]. Conventional LC-MS/MS systems were used, consisting of i-Series UHPLC (Shimadzu, Kyoto, Japan) devices coupled to API 4.000 for ISD analysis or API 4.500 QTrap (AB Sciex, Framingham, MA, USA) for vitamin D analysis. Before being transferred to the conventional LC-MS/MS system, samples containing vitamin D were pretreated using the Freedom EVO®-2 100 Base liquid handling platform (Tecan, Maennedorf, Switzerland). LC-MS/MS based ISD analysis was preceded by manual sample pretreatment; more details can be found in Supplementary Table S6.

The in-house LC-MS/MS method uses calibrators from RECIPE Chemicals & Instruments (Munich, Germany) for daily two-point (vitamin D) and three-point (ISD) calibration curves and deuterated (vitamin D) and analogue internal standards (ISD) (Supplementary Table S6). RECIPE calibrators are traceable to NIST-SRM 972a (25-OH vitamin D), to European Reference Materials ERM-DA110a (tacrolimus), and to Cerilliant-Certified Reference Material CRM C-093 (cyclosporine A). Abbott Alinity has a 6-point calibration curve with monthly calibrations. The manufacturer's 25-OH vitamin D assay is traceable to NIST SRM 2972, the cyclosporine A and tacrolimus calibrators to Abbott's internal reference standards. Continuous quality control management of all comparator methods was performed according to the national law in Germany [34].

Between method comparisons were analyzed by Bland-Altman-plot and regression analysis using the model of Passing-Bablok [35,36].

2.5. Analyzer usability and acceptability

Four laboratory staff members, including beginners and experts in LC-MS/MS methods, were trained as key operators by the manufacturer. After three days of in-house training, each key operator operated the system autonomously for at least one week and completed a structured questionnaire covering various items of user-friendliness and acceptance of the Cascadion™ SM analyzer. Additionally, three other staff members trained by the key operators and two operators at the University Hospital of Tübingen filled out the questionnaire; all without previous LC-MS/MS experience. Each question could be answered on a 10-point scale, with "1" being the worst and "10" being the best score. Informed consent was obtained from participating operators.

2.6. Data analysis

Validation Manager™ software (Finbiosoft, Espoo, Finland) was used to calculate the observed mean, standard deviation (SD), median, correlation coefficient, coefficient of variation (CV), and bias; to conduct Passing-Bablok regression analysis; and to create Bland-Altman analysis plots. Excel® for Microsoft 365 Version 2202 (Microsoft, Redmond, Washington, USA) was used to calculate instrument error frequencies and to evaluate the usability questionnaire.

3. Results

3.1. Verification of analytical performance

Deviations from linearity were within acceptable limits and linearity was shown throughout the entire clinically relevant ranges for 25-OH vitamin D2 ($R = 0.997$; 3.9–110.0 μg/L), 25-OH vitamin D3 ($R = 1.000$; 4.9–115.0 μg/L), cyclosporine A ($R = 0.999$; 20.3–760.0 μg/L) and tacrolimus ($R = 0.998$ 1.9–28.3 μg/L). No carryover was observed

for samples with extremely high concentrations (25-OH vitamin D2 and D3: greater than 100 µg/L; cyclosporine A: 809 µg/L; tacrolimus: 31 µg/L) into subsequently analyzed blank samples.

Within-batch imprecision was ≤ 4.9% for 25-OH vitamin D2, ≤2.5% for 25-OH vitamin D3, ≤6.3% for cyclosporine A and ≤ 6.7% for tacrolimus. Between-batch imprecision and bias over the whole study interval were within allowable levels for all analytes and control levels (Table 1). CV and bias were < 8% and < 3%, respectively, for 25-OH vitamin D2 and D3, and < 7% each for ISDs.

Results obtained on EQA samples showed good linear agreement with the assigned spiked or LC-MS/MS consensus values provided by the EQA provider (R greater than 0.99). The mean bias to the assigned values was –1.6% for 25-OH vitamin D3 (n = 9; all < 15%), –6.0% for cyclosporine A (4 out of 6 samples < 10%), and –7.5% for tacrolimus (8 out of 9 samples < 10%). Only one of the EQA vitamin D samples contained 25-OH vitamin D2, with a bias to the assigned value of –2.0%.

3.2. Inter-laboratory and method comparison

When comparing the results obtained on the Cascadion™ SM analyzer at SYNLAB Leinfelden and an identical one at the University Hospital Tübingen, good correlations were observed with a low median bias for 25-OH vitamin D3 (–4.5%), cyclosporine A (–0.9%), and tacrolimus (1.9%) (Table 2).

Since only two vitamin D samples contained 25-OH vitamin D2, inter-laboratory and method comparisons were made only for 25-OH vitamin D3 and not for total 25-OH vitamin D. This has the advantage of allowing for a direct comparison of the deviation between Cascadion™ SM and in-house LC-MS/MS for 25-OH vitamin D3 to that against the immunoassay, since immunoassays cannot separately measure 25-OH vitamin D2 and 25-OH vitamin D3.

Passing-Bablok regression analysis indicated no constant error for 25-OH vitamin D3 and tacrolimus when comparing automated and in-house LC-MS/MS (Fig. 1, Table 2). In contrast, the confidence interval (CI) of cyclosporine A for the intercept (–18.20 to –8.99 µg/L) does not include 0, indicating a constant error. This has already been reported for the Cascadion™ SM by Fania et al. [18] and may be negligible considering the wide therapeutic range of cyclosporine A. A small proportional error with a CI for the slope near 1 was present for all three tests. These errors are clinically negligible as median bias was below an acceptance limit of < 15% for 25-OH vitamin D3 (–10.6%, 95% CI: –13.4%; –8.7%) and < 10% for cyclosporine A (–4.3%, 95% CI: –7.6%; –1.2%) and tacrolimus (–6.6%, 95% CI: –9.0%; –5.1%).

Excellent correlation with minimal proportional error and acceptable median bias of 6.6% (95% CI: 2.5%; 8.9%) was seen between Cascadion™ SM 25-OH vitamin D3 test and Alinity total vitamin D immunoassay. Because of the few 25-OH vitamin D2 samples, the result would not have been different if total vitamin D Cascadion™ SM results had been used for comparison. However, the bias against the cyclosporine A CMIA was –11.7% (95% CI: –18.6%; –6.1%) and for the tacrolimus CMIA –23.9% (95% CI: –27.3; –19.8), both exceeding the acceptable 10% limit of deviation [28].

3.3. Analyzer usability and acceptability

After a 3-day training session on the analyzer, the laboratory staff was able to operate and maintain the device independently and train other personnel on the device. Six (67%) of the nine operators, including students, technical assistants, and academics of various disciplines, had no, or <1-year, previous experience with LC-MS/MS. Three (33%) of the operators were able to compare the fully automated LC-MS/MS system with a previous experience of conventional LC-MS/MS, four (44%) to automated immunoassay or both, and two (22%) had no experience with either technology.

Overall, all nine test operators who were surveyed stated that their experience with the Cascadion™ SM analyzer was rather good to very good, scoring 6 to 10 on a 10-point scale (with 10 being the highest rating) (Fig. 2). All operators confirmed that the analyzer was easy (scores 7–8) or very easy (scores 9–10) to use, particularly when starting the device and reloading probe wash solutions. Neutral to positive approval was given for ease of changing the cartridge, verifying and accepting results, and measuring priority samples. However, two (22%) out of nine operators also indicated certain problems (scores 1–4) with refilling reagents during operation and reloading solvent bottles for liquid chromatography, possibly due to delayed alerts. The daily maintenance tasks (see Supplementary Table S7), which took about 10 min, were found to be easy by the eight operators who answered the question (89%).

The user interface and presentation of the information on the screen were found to be mostly user-friendly (scores 7–10) by eight (89%) and seven (78%) users, respectively. The status indicators and instrument alerts were understandable for seven (78%) and four (44%) operators, respectively. When help was needed, the on-board help instructions were easily to very easily understandable for six (67%) users. In cases where the instructions were not found to be helpful, the operators gave consistently positive feedback for the manufacturer's service. Remote diagnostics were additionally offered by the manufacturer, but could not be tested during the trial period.

Table 1
CV and bias over the whole study interval.

		expected, µg/L	n	mean, µg/L	SD, µg/L (95% CI)	%CV[a] (95% CI)	%bias (95% CI)
25-OH vitamin D2							
	Control 1	9.5	99	9.5	0.72 (0.63–0.85)	7.6 (6.7–8.9)	0.2 (-1.3–1.7)
	Control 2	28.7	100	28.8	1.79 (1.57–2.10)	6.2 (5.4–7.3)	0.5 (-0.7–1.7)
	Control 3	86.4	100	86.7	4.35 (3.79–5.12)	5.0 (4.4–5.9)	0.3 (-0.7–1.3)
25-OH vitamin D3							
	Control 1	10.2	103	10.2	0.69 (0.61–0.81)	6.8 (6.0–7.9)	-0.1 (-1.5–1.2)
	Control 2	29.4	103	30.1	1.63 (1.42–1.90)	5.4 (4.7–6.3)	2.5 (1.5–3.6)
	Control 3	89.1	103	91.0	4.46 (3.89–5.22)	4.9 (4.3–5.7)	2.2 (1.2–3.2)
cyclosporine A							
	Control 1	16.1	119	16.8	0.67 (0.59–0.79)	4.0 (3.5–4.7)	4.2 (3.4–5.0)
	Control 2	364.1	119	366.7	12.65 (11.10–14.80)	3.5 (3.0–4.0)	0.7 (0.1–1.3)
	Control 3	661.3	119	663.6	21.75 (19.20–25.30)	3.3 (2.9–3.8)	0.4 (-0.2–1.0)
tacrolimus							
	Control 1	2.1	113	2.2	0.14 (0.13–0.17)	6.5 (5.7–7.5)	6.1 (4.8–7.3)
	Control 2	12.6	113	13.3	0.54 (0.48–0.63)	4.1 (3.6–4.7)	5.8 (5.0–6.6)
	Control 3	26.5	113	27.6	1.19 (1.05–1.37)	4.3 (3.8–5.0)	4.0 (3.1–4.8)

[a] $CV_{\text{within-lab}}\ (\%) = \sqrt{CV_{within-run}(\%)^2 + CV_{between-run}(\%)^2 + CV_{between-day}(\%)^2}$.

Table 2
Bias for inter-laboratory and method comparisons of the Cascadion™ SM analyzer using Bland-Altman-analysis and Passing-Bablok regression analysis.

	comparative measurement	n	mean bias, % (95% CI)	median bias, % (95% CI)	intercept (95% CI)	slope (95% CI)
25-OH vitamin D3	immunoassay (Alinity)	95	5.1 (2.2; 7.9)	6.6 (2.5; 8.9)	-0.07 (-1.55; 0.99)	1.08 (1.02; 1.15)
	LC-MS/MS	95	-10.7 (-12.7; -8.6)	-10.6 (-13.4; -8.7)	-0.58 (-1.57; 0.10)	0.92 (0.89; 0.97)
	Cascadion (Tübingen)	94	-4.3 (-5.4; -3.3)	-4.5 (-6.1; -3.3)	-0.51 (-0.89; 0.08)	0.98 (0.95; 0.99)
cyclosporine A	immunoassay (Alinity)	50	-15.1 (-20.0; -10.2)	-11.7 (-18.6; -6.1)	-10.60 (-18.00; -1.77)	0.99 (0.90; 1.05)
	LC-MS/MS	100	-4.2 (-7.8; -0.7)	-4.3 (-7.6; −1.2)	-13.9 (-18.20; -8.99)	1.11 (1.06; 1.17)
	Cascadion (Tübingen)	90	1.4 (-1.0; 3.7)	-0.9 (-2.2; 0.6)	−1.89 (-4.65; -0.37)	1.01 (1.00; 1.05)
tacrolimus	immunoassay (Alinity)	47	-23.4 (-26.9; -19.9)	-23.9 (-27.3; -19.8)	0.75 (0.31; 1.07)	0.67 (0.60; 0.75)
	LC-MS/MS	99	-6.5 (-9.1; -3.9)	-6.6 (-9.0; -5.1)	0.16 (-0.04; 0.39)	0.91 (0.86; 0.95)
	Cascadion (Tübingen)	65	1.8 (0.3; 3.3)	1.9 (0.0; 3.5)	0.10 (-0.03; 0.14)	1.00 (0.99; 1.02)

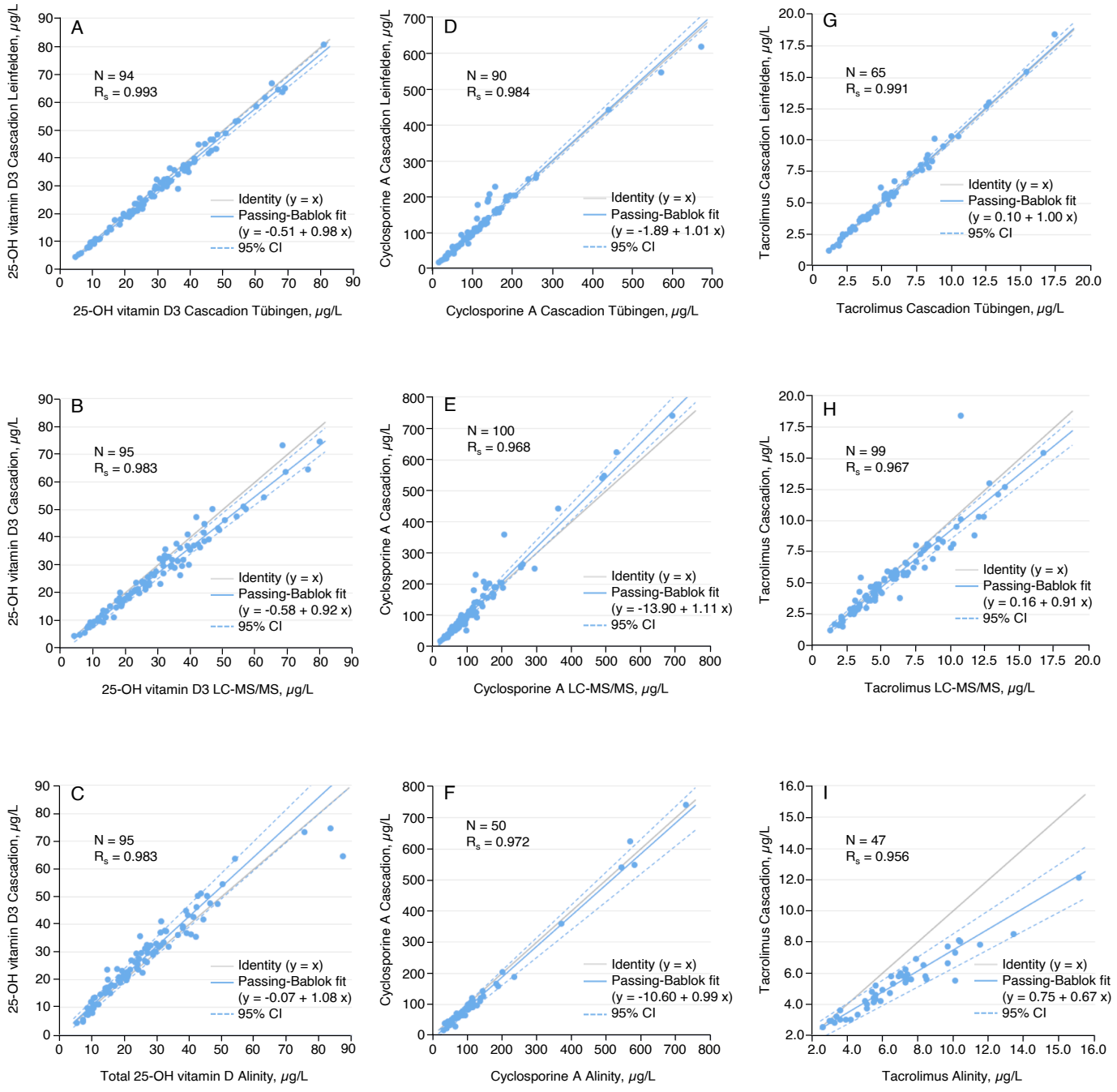

Fig. 1. Passing-Bablok fit of fully automated LC-MS/MS between laboratories, with in-house LC-MS/MS, and with Abbott Alinity CMIA immunoassay for 25-OH vitamin D3 (A-C), cyclosporine A (D-F) and tacrolimus (G-I). The grey line indicates the perfect correlation. R_s, Spearmańs rank correlation coefficient; N, number of samples analyzed.

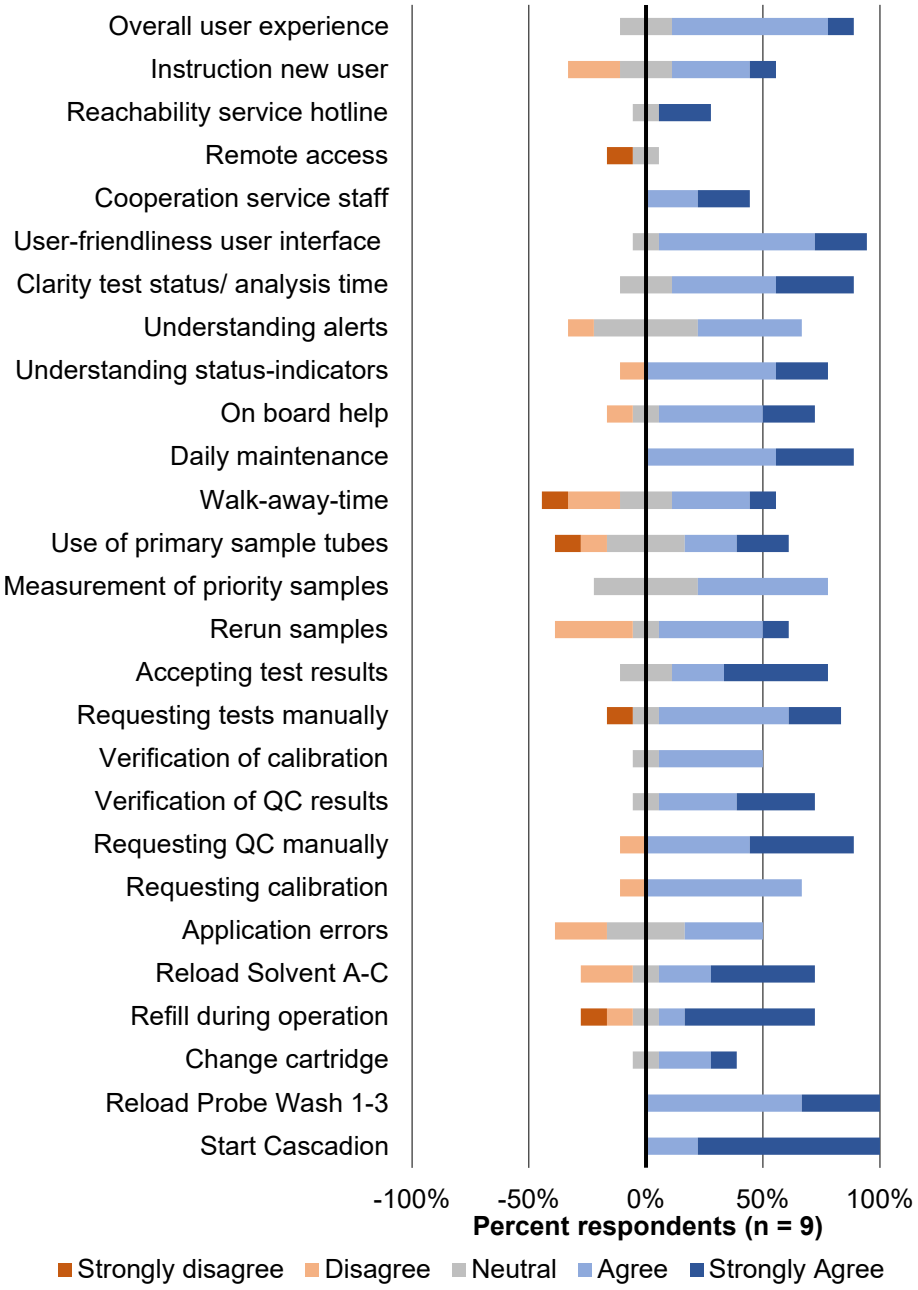

Fig. 2. Survey responses to questions about user-friendliness of the fully automated LC-MS/MS on a 10-point rating scale. Scores 9–10, strongly agree; 7–8, agree; 5–6, neutral; 3–4, disagree; 1–2, strongly disagree.

On the other side, the operators most frequently reported minor or major difficulties (scores 1–4) with short walk-away time (n = 3, 33%) and rerunning samples after an error occurred (n = 3, 33%). Two (22%) operators also indicated problems with the use of primary sample tubes, whose type could not be changed in the instrument for individual samples of a batch run. In addition, two (22%) operators stated that application errors were likely.

Finally, in open questions, the operators were able to state which features of the analyzer they liked best and what they found most troublesome. On the positive side, the simple operation of the analyzer was mentioned most frequently, followed by the user interface, the automatic sample preparation, and the loading of reagents. The following problems bothered users the most: changing sample tube type, errors, delayed warning alerts, and short walk-away times.

The robustness of the system was satisfactory; during the five months of the trial, more than 2000 blood samples were analyzed on the Cascadion™ SM analyzer, generating more than 7000 results. Aspiration errors occurred in121 (7%) out of 1789 samples over 60 documented days. Result errors (e.g., peak quality) occurred in 60 (1%) of 7870 patient sample results, 82 (4%) of 2095 total control results, and 20 (2%) of 864 calibration results (Supplementary Table S8-S10). In most cases, the results could still be accepted. Forty instrument errors were registered on 35 days, corresponding to 24% of 145 documented days. There were 22 different types of instrument errors; 60% of which concerned device control (Supplementary Table S11).

4. Discussion

A wider adoption and routine use of LC-MS/MS in the clinical laboratory is expected to depend on automation and integration into the laboratory information system, as was the case for common immunoassay platforms, which facilitated usability and 24/7 availability

[11,37].

The system tested here, the first "all-in-one" LC-MS/MS system, fulfilled these requirements by streamlining the time-consuming manual steps in the pre- and post-analytical phase of the LC-MS/MS workflow by eliminating them. This included barcode reading of patient samples, automated sample preparation, automation of chromatographic peak review, and bidirectional transfer of test requests and results between the laboratory information system and the LC-MS/MS. This precluded potential errors during pipetting or result transmission by humans. To further increase throughput, two liquid chromatography channels were coupled to the MS/MS, allowing parallel pre-analysis of two assays while fully exploiting the MS detection capacity.

As a result of the five-month trial, a turnaround time of approximately 30 min was achieved from sample loading to the first result, with subsequent results every three minutes. In another study, it was shown that TAT could be reduced by more than half with the fully automated Cascadion™ SM analyzer compared to conventional LC-MS/MS [21]. Timely transmission of results to patients and physicians is particularly important for therapeutic monitoring of ISDs with a narrow therapeutic window or for avoiding overdosing-induced toxicity by vitamin D supplementation [38,39].

The Cascadion™ SM analyzer has already proven to be suitable for 24/7 service in hospital laboratories [16–22]. However, commercial clinical laboratories differ from hospital laboratories in several aspects, the most important being that commercial laboratories are typically only available on workdays and process larger sample batches and receive various types of primary tubes. Thus, high throughput analyzers with long walk-away times for unsupervised runs, including overnight, and a robust operation to avoid having to repeat testing from more difficult to obtain outpatient samples are key issues. Additionally, newly introduced tests must fit into the predefined workflow of commercial laboratories that offer thousands of different tests.

Before introducing the CE IVD-certified, fully automated LC-MS/MS including pre-validated assays for routine diagnostics in our commercial laboratory, we first had to verify that performance specifications, such as precision and accuracy, were met [32]. In this paper, only tests for 25-OH vitamin D, cyclosporin A, and tacrolimus were considered because they are available as immunoassays in our laboratory network for method comparison.

Our results confirmed the excellent analytical performance of the automated system reported in previous studies in hospital laboratories [16–22]. CV and biases were $< 8\%$ for all control levels over the five-month trial for 25-OH vitamin D, cyclosporin A, and tacrolimus. The mean bias from EQA samples was within published accuracy acceptance limits of $< 15\%$ for 25-OH vitamin D3 samples (range –11.0% to 6.0%), and $< 10\%$ for cyclosporine A (range –13.6% to 3.2%) and tacrolimus (range –18.3% to 5.0%) samples. None of the individual results exceeded the permitted deviation of 25% from the assigned values required by the Guidelines of the German Medical Association [34]. Furthermore, linearity was demonstrated, and no carry-over was observed, thus qualifying the assays for routine diagnostics.

The high degree of agreement between Cascadion™ SM systems, even in different laboratories, as reported in previous studies [16,20,21], was also observed between our commercial laboratory and a hospital laboratory, with a median bias of $< 5\%$ for 25-OH vitamin D3, cyclosporine A, and tacrolimus. Thus, automation could further promote result quality beyond the achievements already made by harmonizing LC-MS/MS and immunoassays [10,39,40].

Despite the development of reference measurement procedures, higher-order reference materials, standardization programs, and proficiency-testing schemes for some analytes, such as 25-hydroxyvitamin D, there are still some LC-MS/MS assays that do not comply with performance requirements [7]. For cyclosporine A, there is still no higher-order whole blood reference material recognized by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) [41], which would be preferable to reduce calibration bias.

When comparing measured concentrations between the fully automated LC-MS/MS and accredited in-house LC-MS/MS methods, bias was within acceptable limits for all analytes. Good result agreement was also observed between the Cascadion™ SM 25-OH vitamin D assay and the Abbott Alinity I CMIA immunoassay, with a bias of + 6.6%. This magnitude is similar to a negative bias of –6.5% reported in a previous study comparing the Cascadion™ SM assay with CMIA immunoassay on the Architect platform (Abbott, Chicago, IL, USA) [19]. However, non-analytical factors may influence method selection; for example, the minimum sample volume required for analysis is lower for Abbott CMIA immunoassays than for the Cascadion™ SM analyzer (150 µl versus 285 µl for 25-OH vitamin D and 200 µl versus 350 µl for immunosuppressants). This is more likely to enable analyses of low-volume samples, such as pediatric samples. Other criteria might include dilution options for highly concentrated samples supported by the Abbott Alinity device, but not by the Cascadion™ SM. On one hand, one method can be used as a backup for the other when analyzing 25-OH vitamin D. On the other hand, one should be careful which immunoassay method to choose; another study found comparable results between the Cascadion™ SM assay and an electrochemiluminescence immunoassay (ECLIA), but significant deviations from a chemiluminescent immunoassay (CLIA) [16].

In contrast to the 25-OH vitamin D assay, only moderate agreement was found between the Cascadion™ SM cyclosporine A and tacrolimus tests and the corresponding Abbott Alinity CMIA immunoassays. Higher immunoassay concentrations were also reported by others for cyclosporine A and tacrolimus on the Architect platform (CMIA) compared to conventional LC-MS/MS [42–44], as well as for an antibody-conjugated magnetic immunoassay (ACMIA) relative to the Cascadion™ SM analyzer [17]. A plausible explanation is metabolite cross-reactivity or heterophile antibodies [42,44]. The manual pre-treatment step required for ISD CMIA compared to the 25-OH vitamin D CMIA, as well as the lack of traceability of the ISD assays to higher order reference standards, may have contributed to the poor agreement between ISD CMIA and the fully automated LC-MS/MS.

After a short training period, even non-specialized personnel could operate the fully automated LC-MS/MS device independently, and specialized LC-MS/MS staff could focus on more challenging, non-repetitive tasks. Consistent with a recent survey by Mathieu et al [21], users in our study indicated that the device was easy to use and maintain.

Cost aspects must be considered before deciding to introduce an automated commercial LC-MS instrument into a clinical laboratory. An automated instrument with ready-to-use assay kits may be more expensive than a flexible and versatile LC-MS instrument using in-house prepared reagents and controls. However, it is difficult to make an economical head-to-head comparison of laboratory-developed test (LDT) LC-MS/MS methods and the Cascadion™ SM instrument, as this depends on country- and laboratory-specific circumstances such as the total number of samples, utilization of the instrument, or direct labor time [45]. The typically higher initial hardware investment and operating costs for CE-IVD-certified assays of an automated, ready-to-use instrument may be compensated by 1) lower salary costs as less skilled personnel are needed, 2) less effort for method development, validation, ongoing monitoring and maintenance, and 3) the ability to run the instrument 24 h a day. Around the clock service and a favorable turnaround time due to random access sample processing compared to batch analysis can increase the return on investment by attracting customers. Automated LC-MS may not necessarily be more cost-intensive than automated immunoassays (which rely on expensive antibodies). In addition, LC-MS/MS tests may be better reimbursed by national health insurance systems, depending on the country. To be cost-effective, the instrument must be fully utilized. This is more likely to be the case if there is a large assay selection for different analytes available for the instrument. Although the Cascadion™ SM may have been too expensive for analysis of a few samples per day or for research

laboratories, this is not necessarily true for high throughput commercial clinical laboratories.

Regarding sample processing, there is still potential for improvement, such as integrating a sample dilution option, simultaneous use of tubes with flat and round bottoms, and longer walk-away time for unattended overnight runs. However, these are minor technical weaknesses that do not detract from the improved standardization of analysis and inter-laboratory result harmonization achieved through full automation. Unfortunately, these improvements will not be realized by Thermo Fisher, as the instrument was recently discontinued. Potential reasons for this decision may have been the high price due to a limited number of instruments in the market, the size of the instrument, the lack of integration into a total laboratory automation concept, and the limited assay menu available at the time when it was introduced [46]. Thus, it may not have met the needs of various laboratories; such as academic university hospitals serving transplant centers that require 24/7 therapeutic drug monitoring of ISDs and clinical toxicology; or large commercial laboratories that want to determine hormones and vitamins with high throughput.

However, the device opened a window into the future by adopting many of the features of routine clinical chemistry analyzers. It combined an automated sample reading and preparation system with an LC-MS/MS system and online transfer of results to a laboratory information system. LC-MS assay automation continues to be a hot topic, as evidenced by Roche Diagnostics' intention to launch an analyzer fully integrated into the Cobas family with a much broader assay menu [46]. The road to laboratory automation is irreversible in times of rising laboratory costs and a shortage of qualified personnel. We have observed this trend in general clinical chemistry laboratories and in nucleic acid testing (NAT), where total laboratory automation is now common. An automated LC-MS system should, therefore, meet all the performance requirements of state-of-the-art, high-volume clinical chemistry and NAT analyzers, including robustness, ease of use, and intelligent software both within the instrument and for interconnectivity in a robotic laboratory. Last, but not least, the economic footprint should be within the range of immunoassays when considering the marginal return by weighing the total assay cost including personnel and reimbursement.

5. Conclusions

With more CE-IVD-certified assays available, fully automated LC-MS/MS analyzers would not only benefit hospital laboratories, but also commercial laboratories. However, more new automated LC-MS/MS systems need to be made available in order to accelerate LC-MS/MS automation and its associated benefits in routine laboratories.

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CRediT authorship contribution statement

Sina Junger: Investigation, Formal analysis. **Miriam Hoene:** Methodology, Validation, Writing – review & editing. **Maria Shipkova:** Conceptualization, Writing – review & editing. **Gudrun Danzl:** Resources. **Christof Schöberl:** Investigation. **Andreas Peter:** Resources. **Rainer Lehmann:** Resources, Writing – review & editing. **Eberhard Wieland:** Supervision, Writing – review & editing. **Helmine Braitmaier:** Project administration, Data curation, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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