Article

Clinical Mass Spectrometry in Immunosuppressant Analysis: Toward a Full Automation?

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Abstract: The analysis of immunosuppressive drugs allows the physician to monitor, and eventually correct, immunosuppressive therapy. The panel of molecules under evaluation includes cyclosporine A (CsA), tacrolimus, sirolimus, and everolimus. Initially, assays were performed by immunometric methods, but in the past few years this methodology has been largely superseded by a more accurate and specific technique, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), which is now considered the "gold standard" for immunosuppressant analysis. Both LC-MS/MS and often also immunoassays require a preanalytical manual sample preparation, which involves time-consuming sequential operations whose traceability is often hampered and adds up to the probability of gross errors. The aim of this work was to compare an "open" LC-MS/MS with a fully automated system, consisting of LC instrumentation combined with a triple quadrupole MS, named Thermo Scientific™ Cascadion™ SM Clinical Analyzer (Cascadion). Such automated systems suit the requirements of the reference method and are designed to completely eliminate all of the manual procedures. More than 2000 immunosuppressant samples were analyzed both with the open LC-MS/MS and with Cascadion. Statistics allowed the evaluation of linearity, intra- and inter-assay CV%, bias %, limit of detection and of quantitation, and Passing–Bablok and Bland–Altman plots. Results indicated a good correlation between the two methods. In both cases, methods confirmed their suitability for diagnostic settings. Cascadion could provide support when the presence of specialized personnel is lacking, and/or when great productivity and continuous workflow are required.

Keywords: LC-MS/MS; TDM; automation

1. Introduction

Immunosuppressive therapy is a pharmacological treatment that lowers the activity of the body's immune system [1]. It is used after organ transplantation to reduce the risk of rejection, and for the treatment of autoimmune diseases such as rheumatoid arthritis, myasthenia gravis, and systemic lupus erythematosus [2].

Immunosuppressive therapy involves the use of immunosuppressive drugs (ISDs), active substances able to inhibit the immune system's response. They belong to several classes, among them calcineurin (CaN) inhibitors (cyclosporine A and tacrolimus), and serine/threonine kinase (mTOR) inhibitors (sirolimus–rapamycin- and everolimus). Immunosuppressive drugs used after organ transplant act at different cellular levels to prevent the development of undesirable immune reactions such as antigen presentation, cell activation and proliferation, migration, and tissue infiltration of activated lymphocytes. On the other side, such drugs are responsible for some side-effects mainly related to toxicity. For this reason, therapeutic drug monitoring (TDM) is needed for ISDs to minimize risk of negative side-effects due to the drug toxicity and their narrow therapeutic range.

The goal of the treatment, in fact, is to reduce the risk of graft rejection while avoiding the negative effects related to toxicity (nephrotoxicity, neurotoxicity, diabetes, gastralgia, hyperlipidemia) [2].

From an analytical point of view, initially ISD assays were performed by using immunometric methods which offer high throughput, satisfy the need for automation, and are easy to use. However, it is well known that such techniques are susceptible to interferences [3,4], and are affected by antibody cross-reactivity with drug metabolites, causing an overestimation of ISD concentration, which limits analytical specificity [3,5,6]. In the last 10 years, a growing role was taken by liquid chromatography coupled to mass spectrometry (LC-MS), a more accurate, precise, and specific technique, as it is not affected by interferents and by the presence of inactive ISD metabolites. The features of LC-MS, and in particular of tandem mass spectrometry (LC-MS/MS), allow the simultaneous quantification of a large number of molecules, rapid execution times, and confident quantitative analyses [7,8], which led this methodology to be the gold standard for ISD analysis. On the other side, LC-MS/MS is more complex with regard to instrumental management and usually requires highly specialized personnel, as the main challenge connected to this methodology consists of LC-MS/MS method development and time requirements for proper validation. However, regardless of the technique used, the ISD assays require a pre-analytical preparation of the sample, consisting of the lysis and precipitation of the red blood cells and proteins, in order to collect the ISDs containing supernatant to analyze. ISDs, in fact, are mainly bound to erythrocytes, so this purification step is mandatory [9,10]. Often, these steps are manually performed as well as operations such as numbering/transcription, programming of the work list on the instrument, and the subsequent insertion of the results in the laboratory information system. Beyond being time-consuming, these steps also amplify the probability of gross errors. In ISD analysis, the need for timely, 24/7 diagnostics often collides with the parallel need for "LC-MS grade" analytical precision. Automated analyzers, performing both LC-MS analysis and sample preparation, could potentially fill this gap, integrating the automation in the pre-analytical and analytical management of samples, then improving the efficiency of sample processing, as well as the accuracy and quality of many manual intensive activities, and reducing the risk of injury and exposure to solvents. Furthermore, standardization of sample preparation and better reproducibility of tests would be achieved [7,11].

The organization of clinical laboratories is constantly progressing towards an ideal scenario where laboratory automation replaces boring and repetitive routine steps in order to satisfy the increasing number of diagnostic and research needs [7,11].

LC-MS/MS is starting to benefit from recent automation advances due to the introduction of innovative analytical platforms integrating a number of components including pumps, columns, solvents with undisclosed formulation, calibrator/control materials stabilized in surrogate matrices and pre-packed reagent bottles, robotics for sample handling such as centrifuges, mass analyzers, and systems of waste disposal [7,12]. Moreover, the software should be easily integrated with the LIS (Laboratory Information System) in order to completely eliminate manual operations and enable a seamless traceability of all reagents and materials.

Recently, some automated systems, specifically designed for MS analyses, have been introduced such as the CLAM 2000 (Shimadzu, Kyoto, Japan), a sample preparator connected to LC-MS instrumentation, and the Thermo Scientific Cascadion SM Clinical Analyzer (Thermo Fisher Scientific Oy, Vantaa, Finland), a fully automated and integrated system. The latter provides automated sample preparation with simple procedures for reagent and sample dispensing, mixing, centrifugation, incubation, and automatic transfer to LC-MS/MS [7,12], with the final aim to make LC-MS/MS an integral, technical, feasible, and cost-effective part of medical diagnostics [8].

Given these considerations, the purpose of this work is to compare an "open" LC-MS/MS system (Shimadzu and kit from Recipe, Recipe Chemicals + Instruments GmbH, Munchen, Germany), adopted for routine ISD analysis by our laboratory, consisting of a

Nexera X2 LC coupled to a TripleQuad 8040 MS (Shimadzu), to a fully automated platform, Cascadion SM (Thermo Fisher Scientific, Waltham, MA, USA), consisting of instrumentation with LC technology combined with a triple quadrupole mass spectrometer (therefore suitable for the requirements of the reference method) interfaced with an integrated sample reading, loading, and preparation system, to completely minimize all manual procedures.

The work consists of the parallel analysis of 2022 immunosuppressant samples analyzed by both systems, in order to evaluate the outcome in terms of productivity, reliability, ease of use, and consistency of results, all dropped in a work context of a routine laboratory of a university hospital providing the analysis of more than 8000 ISDs/year.

2. Materials and Methods

2.1. Chemicals

Open LC-MS/MS was carried out using the ClinMass complete kit, advanced for immunosuppressants in whole blood (Recipe). The kit consisted of: solid-phase-extraction (SPE) and analytical columns, mobile phase, internal standards, precipitant, whole-blood calibrator set (4 levels), and 3 whole-blood quality controls.

Automatic LC-MS/MS analysis was carried out using the Immunosuppressant panel kit from Thermo Fisher Scientific. The kit consists of 6 calibration levels (Cascadion SM Immunosuppressants Panel Calibrator Set), 3 quality controls (Cascadion SM Immunosuppressants Panel Control), internal standards (Cascadion SM Immunosuppressants Internal Standard), chromatographic columns (Cascadion SM Quick Connect Cartridge H), and mobile phases for chromatography (A, B, and C), as well as solvents for sample preparation.

2.2. Samples

Tacrolimus (n = 1757), cyclosporine A (n = 203), sirolimus (n = 42), and everolimus (n = 117) assays were performed on a total of 2022 whole blood samples from the Transplant Center and the Withdrawal Center of the "Maggiore della Carità" University Hospital of Novara (Novara, Italy), and from the Transplant Centers of the Hospitals of Biella (Biella, Italy), Borgomanero (Borgomanero, Novara, Italy), Verbania (Verbania, Verbano-Cusio-Ossola, Italy), and Vercelli (Vercelli, Italy).

Samples were collected in BD vacutainer tubes containing K_2EDTA as anticoagulant (Becton Dickinson, Milan, Italy), then they were stored at 4–8 °C until analysis. This study was carried out following the rules of the Declaration of Helsinki of 1975, and all data were properly anonymized.

2.3. Chromatographic and Mass Spectrometry Analyses

Open LC-MS/MS analysis (Shimadzu–Recipe): the tubes were carefully shaken through tube inversion then the erythrocyte membranes were precipitated by adding 220 μL of a mixture of precipitant and labeled internal standards (Recipe) to 100 μL of each sample. Then, samples were vortexed for 1 min, centrifuged at 10,000 rpm for 5 min, and the supernatant was dispensed in vials and 30 μL was subjected to LC-MS/MS analysis by using a Shimadzu Nexera X2 HPLC interfaced with a TripleQuad 8040 mass spectrometer (Shimadzu). The HPLC system was equipped both with a solid-phase-extraction column (SPE) for sample extraction and purification, and with an analytical column (Recipe) maintained at 60 °C. SPE flow rates were set as follows: 0.00–0.01 min at 0.1 mL/min (loading phase), 0.01–0.50 min at 2.5 mL/min (injection phase), 0.51–1.50 min at 0.1 mL/min, 1.51–1.99 min at 2.5 mL/min, and equilibration at 0.1 mL/min until 2.00 min. As regards the analytical column conditions, the chromatographic parameters were set as follows: 0.00–1.30 min at 0.5 mL/min, 1.35–1.55 min at 1 mL/min, 1.65–2.00 min at 0.5 mL/min. Subsequently, the analytes were transferred to MS operating in positive modality (desolvation temperature 220 °C, interface voltage 2500 V, nebulizer gas flow 3 L/min, and drying gas flow 15 L/min), monitoring the transitions indicated in Table 1.

Table 1. MRM transitions adopted for analysis with Shimadzu–Recipe. Monitored analytes are listed together with the corresponding retention time (RT), precursor, and product ions used both for quantification and for qualification, and collision energy (CE).

Analyte	RT (min)	Quantification Ions		Qualification Ions		CE (V)
		Precursor Ion (*m/z*)	Product Ion (*m/z*)	Precursor Ion (*m/z*)	Product Ion (*m/z*)	
CsA	1.42	1219.7	1202.8	1219.7	1184.8	19
D12-CsA	1.42	1232.0	1215.0	1232.0	1197.1	19
Tacrolimus	1.24	821.5	768.4	821.5	576.3	23
13C-D2-Tacrolimus	1.24	824.5	771.5	824.5	579.3	23
Sirolimus	1.29	931.5	864.5	931.5	882.5	18
13C-D3-Sirolimus	1.29	935.6	864.7	935.6	882.2	18
Everolimus	1.31	975.6	908.4	975.6	926.7	19
13C2D4-Everolimus	1.31	981.6	914.7	981.6	932.6	19

Quantitation was achieved by using 4 calibration levels in whole blood and 3 quality controls (Recipe).

Automatic LC-MS/MS analysis (Cascadion, product code 99990000): sample preparation was completely automated starting from the primary tube due to the integration of a liquid handler system equipped with a centrifuge, a couple of LCs working in "duplex" modality, and a triple quadrupole MS [13–15]. Briefly, after tube shaking, the internal standards were added to the sample. Subsequently, samples were mixed by the stirrer, precipitated by Thermo Scientific precipitant solutions, centrifuged, and the supernatant was injected into one of the 2 LC channels. Chromatography was carried out by using an easily exchangeable module consisting of a pre-purification column (TurboFlow technology, Thermo Fisher Scientific), operating in an aqueous solvent mix, coupled to an analytical column set (Quick Connect Cartridge, Thermo Fisher Scientific) where analytes were separated using an organic gradient (phase B). Subsequently, analytes were transferred to the MS whereas the TurboFlow column was cleaned using solvent C and then reconditioned with a mix of solvent A and B before next injection on the respective channel. Mass transitions were not accessible in the Cascadion software. Quantitation was achieved by using 6 calibration levels and 3 quality controls.

2.4. Statistics

Analyse-it software was used for statistical analysis. In particular, the regression analysis was performed by reporting the values obtained with the Cascadion analyzer (*y*-axis) as a function of those obtained with the open LC-MS/MS method (*x*-axis), together with the identity line ($y = x$), and the regression line built using the non-parametric Passing–Bablok model.

With the same software, the bias was evaluated by the Bland–Altman plot. The latter was constructed by reporting the percentage differences between the two methods as a function of the concentrations of each analyte expressed as the average of the two methods under examination.

3. Results and Discussion

3.1. Sample Preparation and Analysis

Two systems based on the gold-standard methodology, LC-MS/MS, for the analysis of immunosuppressants (cyclosporine A, tacrolimus, sirolimus, and everolimus) in whole blood were compared. In particular, the manual sample preparation followed by multiparametric LC-MS/MS analysis (Shimadzu–Recipe) was compared to a recently introduced fully automated technology (Cascadion). The comparison was carried out analyzing in parallel 2022 blood samples for a total of 2119 assays. Few patients, in fact, were subjected to therapy consisting of administration of multiple ISDs, for this reason a small number of samples were tested for multiple analytes.

With regard to Shimadzu–Recipe, the cumulative chromatogram of tacrolimus, cyclosporine A, sirolimus, and everolimus (Figure 1) shows all of the used mass transitions, and the corresponding retention times for each substance. Representative chromatograms obtained with the Cascadion are also shown for each analyte (Figure 1).
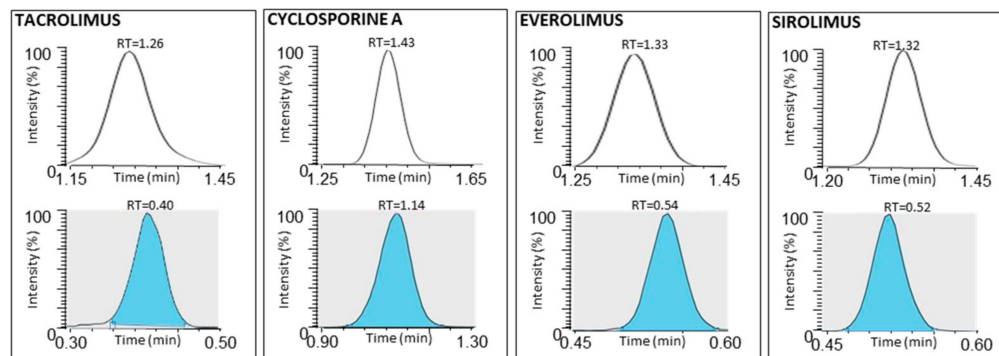

Figure 1. Upper chromatograms: representative chromatograms of level 4 calibrator (tacrolimus = 21.50 ng/mL; CsA = 483.00 ng/mL; everolimus = 22.60 ng/mL; sirolimus = 22.70 ng/mL) used for immunosuppressant quantitation obtained by Shimadzu–Recipe analysis. Lower chromatograms: representative chromatograms of level 2 calibrator (tacrolimus = 2.19 ng/mL; CsA = 24.85 ng/mL; everolimus = 1.98 ng/mL; sirolimus = 1.90 ng/mL) used for immunosuppressant quantitation obtained by Cascadion.

MS data show good peak shape and separation in both cases, despite the different chromatographic conditions (isocratic conditions vs organic gradient).

In the Shimadzu–Recipe method, the calibration is performed once a day with the use of four calibration levels. Each calibration point has an analytical run of 2 min, for a total of about 8 min per analytical session, that is, 4 h/month (where 1 month = 30 working days). Cascadion performs calibration every 30 days using six calibration points analyzed in duplicate on each LC channel, then the duration of the calibration is approximately 18 min/month. Hence, the time for calibration provided by the automated method is largely lower with respect to those provided by the open LC-MS/MS. Table 2 shows an estimate of the times required by each of the two methods for both preparation and calibration, and analysis of 50 samples per day.

Table 2. Times for preparation, calibration, and analysis of 50 samples/day are shown both for Shimadzu–Recipe and for Cascadion.

	Sample Preparation	**Calibration**	**Sample Analysis**
Shimadzu–Recipe	~1 h, calibrators and controls included (manual operation).	8 min/day	100 min/day
Cascadion	30 min for the first result, then a result at ~2.5 min intervals.	~18 min/month	75 min/day

3.2. Method Performance Evaluation

The lower detection limit (LLOD), the lower quantitation limit (LLOQ), and the linearity range of each analyte for both technologies are reported in Table 3.

LLOD and LLOQ were found to be higher in Cascadion. Despite this, for both methodologies the values of LLOD and LLOQ are suitable to determine the immunosuppressant levels according to the therapeutic range proposed by the recently published consensus recommendations [16], but also to the ranges built on the patient population affering to the "Maggiore della Carità" University Hospital of Novara.

Moreover, in Tables 4 and 5, intra-assay and inter-assay precision, and accuracy as bias %, measured on three concentration levels for each analyte, are reported. Both platforms

showed low intra-assay CV%: both for Shimadzu–Recipe and Cascadion, the intra-assay CV% is lower than 4.9%. Moreover, with regard to the inter-assay CV%, in Shimadzu–Recipe it was lower than 5.6%, whereas for Cascadion it was lower than 6.3%. Both methodologies provide CV% fully suitable for laboratory and clinical needs whereby the fully automated system shows even lower CV%.

Table 3. LLOD (Lower Limit of Detection), LLOQ (Lower Limit of Quantitation), and linearity are shown both for Shimadzu–Recipe and for Cascadion, together with the corresponding times for calibration and analysis of 50 samples per day for a total of 30 days.

	Analyte	LLOD [ng/mL]	LLOQ [ng/mL]	Linearity [ng/mL]
Shimadzu–Recipe	CsA	1.75	5.26	5.6–1713.0
	Tacrolimus	0.04	0.13	0.130–60.9
	Sirolimus	0.05	0.15	0.150–67.6
	Everolimus	0.05	0.15	0.150–67.7
Cascadion	CsA	2.83	8.5	8.5–920.0
	Tacrolimus	0.28	0.85	0.85–34.5
	Sirolimus	0.28	0.85	0.85–34.5
	Everolimus	0.28	0.85	0.85–34.5

Table 4. Intra- and inter-assay precision and accuracy (given as bias %) regarding Shimadzu–Recipe analysis are shown for each control level (I–III).

Analyte	Control Level	Assigned Concentration [ng/mL]	Experimental Mean Concentration [ng/mL]	Intra-Assay CV (%)	Inter-Assay CV (%)	Bias (%)
CsA	I	58.40	59.40	2.4	5.5	1.7
	II	118.00	120.34	2.4	4.0	2.0
	III	232.00	251.10	1.4	2.1	8.2
Tacrolimus	I	3.67	3.81	2.8	4.7	3.9
	II	7.69	7.85	3.1	4.5	2.1
	III	15.50	15.49	2.4	4.2	−0.1
Sirolimus	I	4.08	4.18	4.6	5.1	2.5
	II	12.70	12.69	4.1	5.6	<0.1
	III	21.70	21.32	3.3	4.7	−1.7
Everolimus	I	3.84	3.96	4.9	4.8	3.1
	II	12.50	12.58	3.5	3.1	0.7
	III	20.50	20.32	3.5	3.7	−0.9

Table 5. Intra- and inter-assay precision and accuracy (given as bias %) regarding Cascadion analysis are shown for each control level (I–III).

Analyte	Control Level	Assigned Concentration [ng/mL]	Experimental Mean Concentration [ng/mL]	Intra-Assay CV (%)	Inter-Assay CV (%)	Bias (%)
CsA	I	15.43	15.95	1.5	2.9	3.4
	II	374.64	375.94	1.0	2.9	0.3
	III	644.44	644.94	1.1	3.0	2.2
Tacrolimus	I	2.03	2.10	4.9	5.6	3.7
	II	12.50	12.80	3.2	4.5	2.4
	III	25.22	25.82	2.9	4.3	2.4
Sirolimus	I	2.06	2.08	4.8	6.3	0.8
	II	12.34	12.59	4.0	5.8	2.0
	III	24.22	25.62	3.4	5.1	5.8
Everolimus	I	1.93	2.05	4.6	5.5	6.4
	II	12.13	12.40	4.4	5.4	2.2
	III	24.55	25.21	4.5	5.7	2.7

Furthermore, maximum bias observed in Shimadzu–Recipe methodology was 8.2% for CsA whereas that observed in Cascadion was 6.4% for everolimus analysis. In both cases, all observed values are suitable for a clinical setting.

In Figure 2 and Table 6, the correlation plots for each analyte are shown, together with the number of samples analyzed, the equations of the fitting plots, and the confidence intervals (CI).
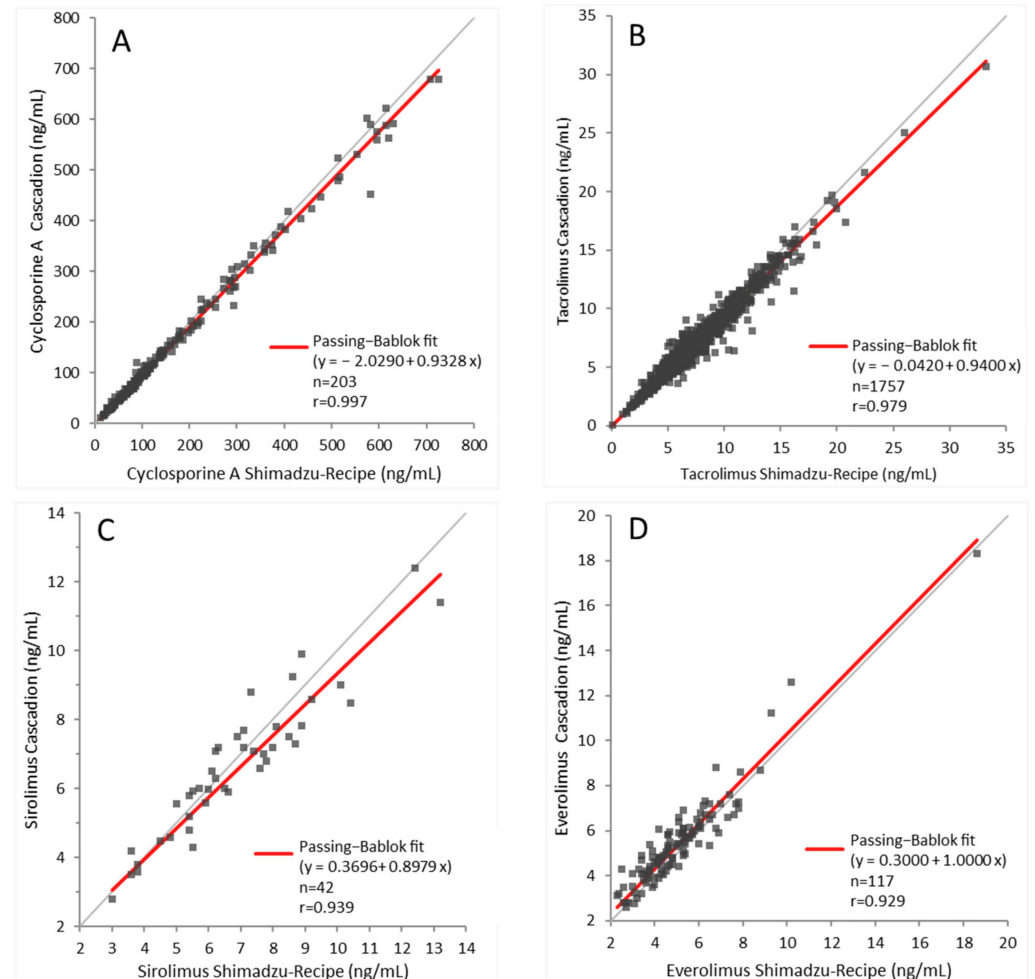

Figure 2. Passing−Bablok fit (red line) for cyclosporine A (**A**), tacrolimus (**B**), sirolimus (**C**), and everolimus (**D**). Grey line indicates the perfect correlation (slope = 1, intercept = 0). Each plot is coupled to regression results.

Table 6. For each analyte, mean (standard deviation) and Passing–Bablok regression parameters are shown both for the reference and test method.

Analyte	N	Shimadzu−Recipe Mean (SD) [ng/mL]	Cascadion Mean (SD) [ng/mL]	Intercept (95% CI) [ng/mL]	Slope (95% CI)
CsA	203	165.62 (158.68)	158.27 (152.18)	−2.03 (−3.07 to −1.13)	0.96 (0.95 to 0.98)
Tacrolimus	1757	7.09 (2.85)	6.65 (2.67)	−0.04 (−0.09 to 0.02)	0.94 (0.93 to 0.95)
Sirolimus	42	6.85 (2.22)	6.68 (2.04)	0.37 (−0.30 to 1.14)	0.90 (0.78 to 1.04)
Everolimus	117	5.03 (2.00)	5.44 (1.97)	0.30 (−0.10 to 0.89)	1.00 (0.90 to 1.09)

A good correlation between the two methods was observed. Moreover, it can be noted that for all four analytes there are strong correlation values without significant variations from linearity as the correlation coefficients for cyclosporine A (0.997), tacrolimus (0.979),

sirolimus (0.939), and everolimus (0.929) show. A good correlation, however, was expected as both the methodologies are based on LC-MS/MS analysis which is the gold standard for immunosuppressant analysis.

Furthermore, the correlation plots demonstrate the absence of constant error for tacrolimus, sirolimus, and everolimus as the relative confidence intervals include 0 for the intercept. The CI of cyclosporine A is comprised between -3.070 and -1.130, hence does not include 0, indicating the presence of a constant error. However, given the wide pharmacological range used for cyclosporine A [16] and the value found for the intercept, that is, -2, this finding can be considered negligible from a clinical point of view.

With regard to the proportional systematic error, CIs of sirolimus and everolimus do include 1 for the slope, indicating the absence of proportional systematic error. The confidence intervals of tacrolimus and cyclosporine A are near 1 (0.9483 and 0.9773, respectively) but do not include this value, indicating the presence of a (minimal) proportional systematic error. This result can be explained by considering the number of samples tested for tacrolimus (n = 1757) and cyclosporine A (n = 203), which is clearly higher than the number of samples tested for everolimus and sirolimus (n = 117 and n = 42, respectively). For this reason, these minimal proportional systematic errors, indicated by the regression analysis, even statistically significant, do not hamper the use of the method and are not relevant from a clinical and a therapeutic point of view.

Finally, Bland–Altman plots were adopted to evaluate the systematic error (Figure 3). The latter was obtained by calculating the bias, which is the mean of all the differences and the relative 95% confidence interval. For each one of the immunosuppressants, no significant systematic errors were detected (all CIs include 0), nor were there particular concentration-dependent trends, as the differences between the two methods randomly accumulate around zero at each concentration level. In particular, 95% of the differences between the two methods are included in the following intervals: -22.0% to 9.0% (tacrolimus), -20.5% to 9.7% (cyclosporine A), -22.7% to 17.4% (sirolimus), and -17.8% to 33.0% (everolimus).
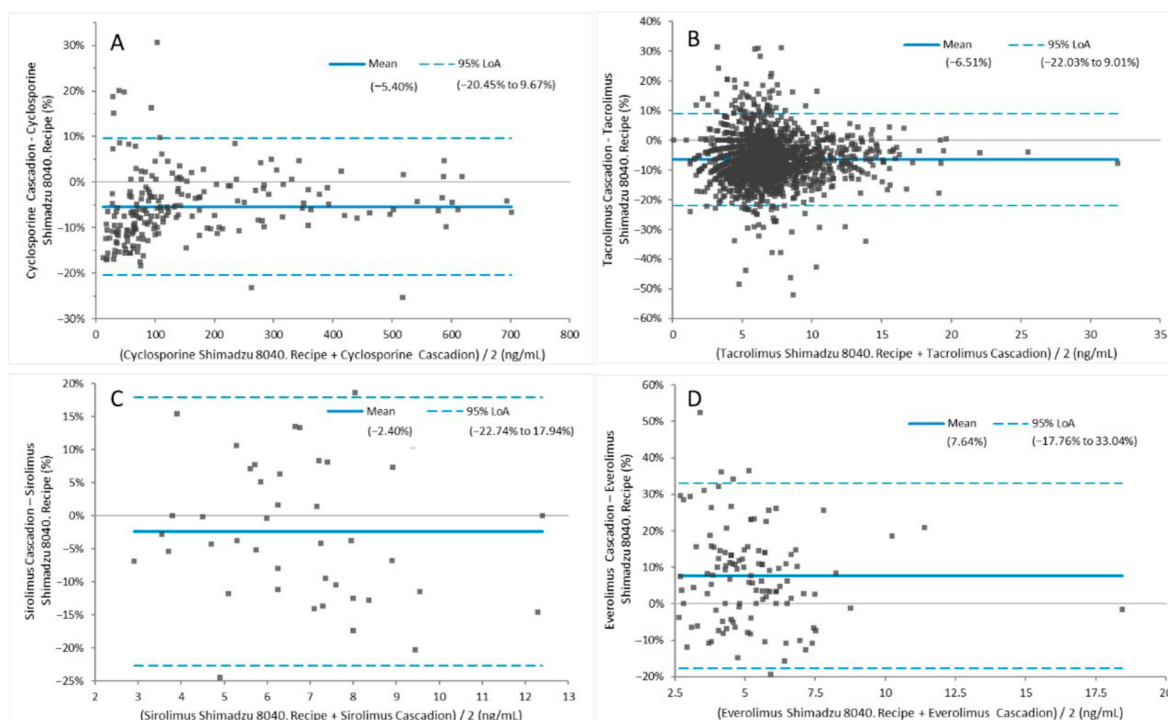

Figure 3. Bland−Altman plot for cyclosporine A (**A**), tacrolimus (**B**), sirolimus (**C**), and everolimus (**D**). In each plot, the mean and the limit of acceptance (LoA) are indicated.

4. Conclusions

A methodology based on manual sample preparation followed by multiparametric LC-MS/MS analysis was compared to a recently introduced fully automated platform. The comparison was carried out analyzing in parallel 2022 blood samples for a total of 2119 assays.

Cascadion shows several advantages, being equipped with a fully automated sample preparation system that reduces time and errors in the pre-analytical phase [11]. Furthermore, there is a high saving of consumables, as the calibration is performed monthly, compared to those performed daily with an open LC-MS/MS system. Moreover, a further advantage is not requiring specific LC-MS/MS training which is usually required by MS technical staff.

With regard to the analytical conditions, neither the different preparation methods nor the different chromatographic conditions seem to impact on the analytical performance.

Cascadion offers a greater precision than Shimadzu−Recipe; however, both methods provide coefficients of variation suitable for diagnostic settings.

Constant, proportional, and systematic errors are absent both for sirolimus and everolimus, whereas tacrolimus seems to be affected by proportional systematic error and cyclosporine A by both kinds of errors. However, as previously stated, although statistically significant, these errors are negligible from a therapeutic and clinical point of view.

Concluding, Cascadion could provide a valuable support when the presence of specialized personnel is lacking, and/or when great productivity is required, due to its good precision and almost-absent operator intervention. In our opinion, Cascadion is suitable for clinical laboratories which need high throughput and a 24/7 diagnostic setting, with a continuous workflow and/or poorly specialized personnel. Of course, adopting a fully automated system would require a sort of "change of perspective" as instrumentation is conceived as a "closed box" and daily operations, such as calibration, are normally performed "only" once a month. This point should be also evaluated considering that integrated LC-MS is conceived with the view of being a ready-to-use tool not subjected to switching-on-and-off procedures, which can have an impact on instrumental stability, and then could involve a daily calibration.

On the other side, open LC-MS/MS better meets the analysis of smaller batches of samples or samples randomly distributed within the working day, and it requires highly qualified personnel, who are often absent in a 24/7 setting.

Furthermore, the chance to directly link the LC-MS platform to the laboratory informatic software, not evaluated in the present work, could greatly improve the diagnostic workflow.

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Institutional Review Board Statement: Ethical review and approval were waived for this study as the proposed paper shows the advancement of a standard practice and does not include a new method, as the technology (based on liquid chromatography coupled to mass spectrometry) is well known, and all the adopted kits were already validated by the manufacturers. Moreover, all the analyses were part of a clinical laboratory practice and were performed only for the scope of drug-treatment monitoring prescribed by physicians [17].

Informed Consent Statement: Patient consent was waived as it is not required: it is implied that informed consent was previously given for the scope of the treatment) [17].

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