



Short communication

Ultra-high throughput dual channel liquid chromatography with tandem mass spectrometry for quantification of four immunosuppressants in whole blood for therapeutic drug monitoring



Tanja R. Zijp^a, Kai van Hateren^a, Hiltjo Kuiper^a, Erwin M. Jongedijk^a, Daan J. Touw^{a,b,*}

^a University of Groningen, University Medical Center Groningen, Department of Clinical Pharmacy and Pharmacology, Hanzeplein 1, 9700 RB Groningen, the Netherlands

^b University of Groningen, Department of Pharmaceutical Analysis, Groningen Research Institute of Pharmacy, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, the Netherlands

ARTICLE INFO

Article history:

Received 18 January 2023

Revised 28 April 2023

Accepted 18 May 2023

Available online 20 May 2023

Keywords:

Dual channel chromatography

Dual LC MS MS

Ultrahigh-speed

Productivity optimisation

Optimal MS MS utilisation

ABSTRACT

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) is the golden standard for immunosuppressants analyses, where optimising throughput by parallel chromatography can reduce costs and turnaround time. We aimed to double our system throughput using a dual LC-MS/MS setup. Therefore, two independent UPLC systems were hyphenated to one triple quadrupole MS, with staggered injections from one autosampler on alternating columns. The method simultaneously measured the analytes tacrolimus, sirolimus, everolimus, and cyclosporin A in whole blood using isotope dilution, with a run time of 1.5 min. Using the dual LC-MS/MS system, net run-to-run time improved from 2.3 to 0.98 min per injection, where throughput increased from 26 to 61 injections per hour. For Performance Qualification, 1101 clinical samples were measured on the dual LC-MS/MS system in addition to the standard system, during a period of one month, and the results were compared using Passing Bablok regression and Bland Altman analysis. There was excellent agreement for all four analytes, with regression slopes of 0.98–1.02x and intercepts of -0.11–0.88 µg/L. Minor bias was demonstrated between the systems with mean differences from -0.93 to 1.43%. In conclusion, the throughput was doubled and idle MS time was reduced with good agreement to the standard system. Currently, the method is applied for clinical routine with frequent peak intensities of >180 injections per day.

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1. Introduction

Immunosuppressive agents are crucial to prevent rejection in solid organ transplant recipients and to preserve organ function. For most immunosuppressive drugs, including tacrolimus, sirolimus, everolimus, and cyclosporin A, frequent monitoring is required [1]. LC-MS/MS is appointed as the golden standard, as it is more sensitive than immunoassays and shows no cross-reactivity with metabolites [2,3]. Advances in methodology and instrumentation have improved the analysis for immunosuppressants in the last years, focusing on improvement in resolution, sensitivity and turnaround time.

As our hospital is one of the major transplant centres in The Netherlands, monitoring of immunosuppressants is a crucial part

of patient care and research. Analyses are performed using LC-MS/MS in whole blood or in dried blood spots [4–8]. The analysis runs 7 days a week with multiple shifts per day, comprising >700 injections per week with peak intensities of over 180 injections per day. The sample load is increasing, the space for new apparatus is limited, and earlier efforts already optimised the run time of the method to 1.5 min. Hence, we looked into system options to increase sample throughput even further, where we focused on decreasing the idle time of the mass spectrometer in between the runs.

Parallel chromatography was our solution to optimise MS time, where two separately working UPLC systems were connected to one MS. In short, all samples are placed in one autosampler and two individual working injectors bring the samples automatically and alternately onto the columns in a staggered way. Accordingly, the analytes from the second column elute successively to the window of detection of the first column. Therefore, throughput of the analysis can double without complex changes to the analyt-

* Corresponding author at: University Medical Center Groningen, Hanzeplein 1 (Internal Postcode AP50), 9700 RB Groningen, the Netherlands.
E-mail address: d.j.touw@umcg.nl (D.J. Touw).

ical method or workflow. This concept has been reported in some configurations in literature [9–11]. However, to our knowledge this concept has not yet been described for any drugs that need therapeutic drug monitoring.

This study describes the Performance Qualification and advantages of an ultra-fast, robust and sensitive dual UPLC-MS/MS system to quantify four immunosuppressants, being tacrolimus, sirolimus, everolimus, and cyclosporin A, with a validated method, for therapeutic drug monitoring.

2. Materials and methods

2.1. Reagents

Tacrolimus, sirolimus, cyclosporin A, and ascomycin were purchased from Cerilliant (Round Rock, TX, USA). Everolimus and [$^2\text{H}_{12}$]-cyclosporin A were acquired from Alsachim (Illkirch Grafenstaden, France). Stock solutions were prepared from the solutions (all 1000 mg/L) in methanol and stored at -20°C . All mobile phase reagents were of analytical grade quality, where methanol was purchased from Biosolve (Valkenswaard, The Netherlands), while formic acid and zinc sulfate heptahydrate were from Merck (Darmstadt, Germany). Ammonium formate was purchased from Thermo Fisher Scientific (NJ, USA). Fresh ultrapure water was obtained from a Milli-Q system (Millipore Corporation, Bedford, MA, USA).

2.2. Sample preparation

Sample preparation was done as reported earlier with minor modifications [8]. In short, 200 μL whole blood was precipitated by addition of 500 μL internal standard solution (containing 100 $\mu\text{g/L}$ ascomycin and 100 $\mu\text{g/L}$ [$^2\text{H}_{12}$]-cyclosporin A in methanol) in a HPLC screw top 1.5 mL glass vial (Fisher Scientific, Houston, Texas, USA). Further precipitation was acquired by adding 25 μL 2.4 mol/L zinc sulfate. All samples were vortexed for 1 min after precipitation and kept at -20°C for at least 10 min. The samples were then vortexed for 1 min, centrifuged at 9500 g for 5 min to form a pellet, and the glass vials were placed in the autosampler. Subsequently, 2 μL supernatant was automatically aspirated and injected onto one of the two UPLC columns.

2.3. Equipment and conditions

2.3.1. Dual LC-MS/MS

The dual LC-MS/MS system consisted of a Vanquish Dual Split Sampler, leading to two Vanquish Horizon pumps with two Accucore C18 2.6 μm 50 $\times$ 2.1 mm columns in one column compartment, which were both attached to a TSQ Quantiva Mass Spectrometer. All apparatus were from Thermo Fisher Scientific (Waltham, MA, USA).

The mobile phase had a constant flow rate of 1.00 mL/min, and a gradient of 0.02 mol/L ammonium formate buffer (pH 3.5, mobile phase A) and methanol (mobile phase B) was applied. For a more detailed description of the gradient, see Table 1. At 0.75 min after sample injection, the flow was switched from waste line to MS line, with the detection window lasting 0.6 min. The detection window included the retention time of all analytes. In total, the time of gradient application was 1.5 min, followed by stabilisation time in which the next injection could take place, of which the preparation usually lasted under 0.5 min. Column temperature and pre-heater in the UPLC were set at 60°C .

The mass spectrometer was operated in positive ionisation mode with heated electrospray ionisation (H-ESI). Selection in the first quadrupole was on the ammonium adducts of the molecule $[\text{M}+\text{NH}_4]^+$, followed by selection on their single charged ions

Table 1

Gradient elutions for dual LC-MS/MS and for the standard system.

Time (min)	Flow (mL/min)	Elution ^a	
		A (%)	B (%)
0.00	1.00	70	30
0.30	1.00	70	30
0.31	1.00	27	73
0.95	1.00	22	78
0.96	1.00	5	95
1.25	1.00	5	95
1.26	1.00	70	30
1.50	1.00	70	30
Until next injection	1.00	70	30

^a A, Ammonium formate buffer 0.02 mol/L (pH 3.5) B, Methanol.

$[\text{M} + \text{H}]^+$ in the second quadrupole. The set parameters for mass spectrometer selection and other relevant parameters are mentioned in Table 2. Furthermore, static ionisation spray voltage was set on 4500 V, and the arbitrary parameters for gas were 50 for sheath gas, 25 for auxiliary gas and 0 for sweep gas. High-purity nitrogen was used for the source gas flows, and high-purity argon was used for collision gas flows. The ion transfer tube temperature was 140°C , and the vaporiser temperature was 350°C .

For system control and data processing, ARIA MX software version 2.6.13 (Agilent, Santa Clara, CA, USA) was used.

2.3.2. Standard LC-MS/MS system for comparison

The standard LC-MS/MS system consisted of a Vanquish Horizon Binary Pump, a Vanquish Autosampler and an Accucore C18 50 $\times$ 2.1 mm column with 2.6 μm solid-core particles within a column compartment, leading to a TSQ Quantiva Mass Spectrometer, all from Thermo Fisher Scientific (Waltham, MA, USA). The analytical method used, including mobile phase parameters and mass spectrometer settings was the same as described in paragraph 2.3.1. However, the standard system idled during stabilisation and injection, resulting in a run-to-run time of 2.3 min.

2.4. Method

The method as described here was an evolution of the previously published LC-MS/MS method [8]. A partial method validation was performed with every hardware update and method iteration, in accordance with the guidelines for bioanalytical methods of both the European Medicines Agencies (EMA) and United States Food and Drug Administration (FDA), and in line with recommendations of the Global Bioanalysis Consortium Harmonisation team [12–14]. Therefore, previously published validation results apply to the current method, and the partial validation results of the currently used iteration are shown in Appendix A.

For the Performance Qualification, response factors were used at previously validated HLOQ concentrations, being 40 $\mu\text{g/L}$ for tacrolimus, sirolimus, and everolimus, and 1600 $\mu\text{g/L}$ for cyclosporin A. The use of response factors instead of calibration curves reduces the amount of overhead samples, thus minimising analysis time in each routine analytical batch.

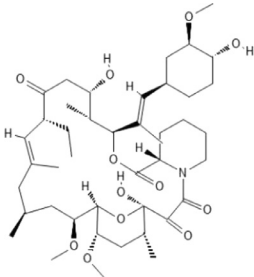
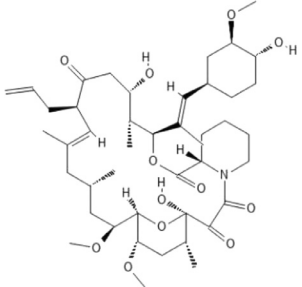
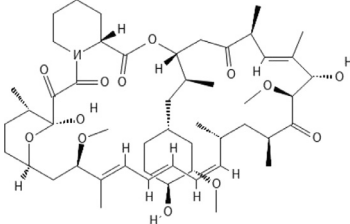
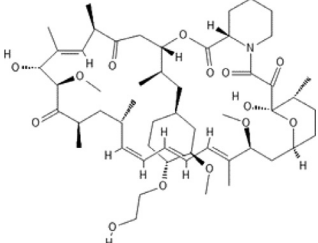
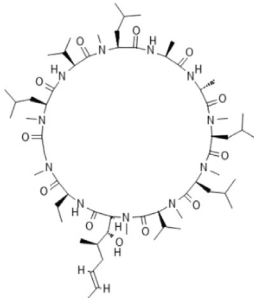
As the use of residual patient material for the Performance Qualification was part of the original purpose for which the patient material was collected, study registration was not required.

2.4.1. Comparison of dual LC-MS/MS to standard

As the same method and the same system components were used for the dual LC-MS/MS, there were no requirements for partial validation according to the EMA/FDA recommendations. However, in accordance with ISO 15189, a Performance Qualification

Table 2

MS transitions for all analytes, including information on structure, retention time, collision energy and radio frequency (RF) lens.

Analyte	Retention time (min)	Collision energy (eV)	RF lens (V)	Mass transition (m/z)
Ascomycin (IS)	0.90	20	92	809.5 > 756.4
				
Tacrolimus	0.93	20	82	821.5 > 768.4
				
Sirolimus	1.00	15	83	931.5 > 864.4
				
Everolimus	1.02	16	88	975.5 > 908.4
				
Cyclosporin A	1.24	15	93	1219.8 > 1202.7
				
[$^2\text{H}_{12}$]-Cyclosporin A (IS)	1.24	15	93	1231.8 > 1214.8

was performed. In a period of one month, multiple batches of routine clinical samples were analysed by the new dual LC-MS/MS setup in addition to the standard system with single LC-MS/MS, and their agreement was evaluated with predefined criteria. According to stricter criteria for cross-validation for immunosuppressant analyses in our laboratory, that were set up by a multidisciplinary team of e.g. transplant physicians, pharmacists, and technicians, the agreement between the methods should be within 15% of the mean for at least 80% of the samples [4]. Also retention

times were compared between the standard and dual LC system, where a bias less than 15% was accepted.

2.5. Quality control

The laboratory participates in the LGC proficiency testing program (LGC Group, Bury, UK) to establish whether accuracy, precision and specificity are acceptable for routine therapeutic drug monitoring, and to evaluate sources of error. The test frequency is

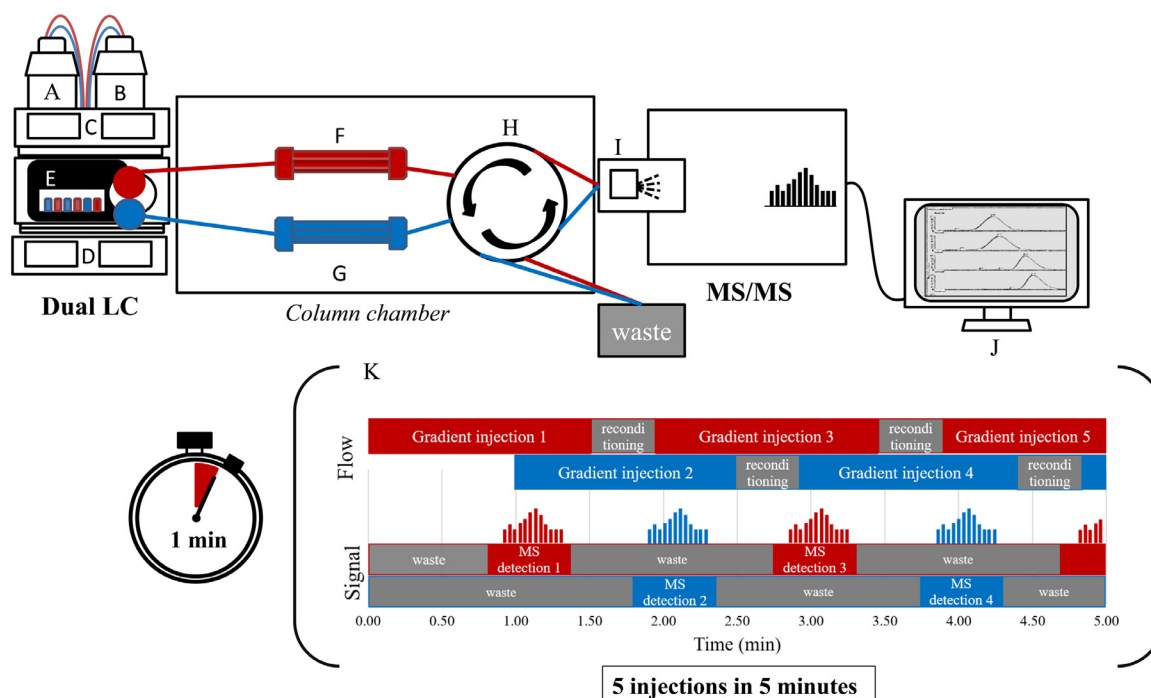


Fig. 1. Schematic representation of the dual LC-MS/MS system. The two mobile phases (A & B) are mixed by two independent binary pumps (C & D) into gradients. The two gradients are led to one autosampler (E) with two independently working injection valves, that inject alternately and staggered onto two columns (F & G). After column separation, the mixtures are led to a 6-port divert valve (H). This valve switches between waste or MS: when analyte of interest washes off the column, the flow is automatically switched to the MS line. Following electrospray ionisation (I), and detection, the signal is shown on a monitor (J). K shows the timeframe of gradient flows and detected signals from the two separate columns with 5 injections in 5 min.

12 times per year for tacrolimus, sirolimus and cyclosporin A, and 6 times per year for everolimus, with 3 samples per test. Measurement uncertainty is calculated as $MU=2 \sqrt{(\text{Bias}_{\%}^2 + \text{RSD}_{\%}^2)}$ and is deemed acceptable under 15% [15].

2.6. Statistics

Agreement between the system configurations was analysed with Passing-Bablok regression and Bland-Altman bias estimation. Both were assessed using Analyse-it version 5.81 for Microsoft Excel (Analyse-it Software Ltd., Leeds, UK). For retention times, the % bias was calculated relative to the standard LC.

3. Results

3.1. Dual LC-MS/MS design

The MS in this design is used efficiently, as idle time is decreased by utilisation of two parallel UPLC systems, which resulted in a throughput of 61 injections per hour and a net run-to-run time of 0.98 min.

As elucidated in Fig. 1, the mobile phase continuously flows to the MS and the “waste” line. During the expected elution of the analytes, the UPLC flow channel is connected to the MS for detection. After the detection window of column 1, where the first UPLC needs reconditioning and is connected to the “waste line”, the MS is available to the analytes separated in column 2. All synchronisations are carried out by the system software.

3.2. Comparison of dual LC-MS/MS to standard

The Performance Qualification was performed by evaluation of agreement between dual and standard LC-MS/MS system configurations.

Therefore, a total of 1101 clinical samples were measured for routine care in 44 unique runs over 17 separate days, including 980 samples for tacrolimus, 70 for sirolimus, 46 for everolimus and 78 for cyclosporin A. Mean concentrations of the patient samples measured were 6.95 µg/L (range: 0–29.00 µg/L) for tacrolimus, 5.40 µg/L (range: 0–20.78 µg/L) for sirolimus, 6.33 µg/L (range: 2.49–21.35 µg/L) for everolimus and 141.0 µg/L (range: 11.0–965.2 µg/L) for cyclosporin A. Therefore, all samples were measured within the linear range. Representative chromatograms of standards and patient samples are shown in Appendix B.

No significant differences were found between the systems, with 99.99% of the repeated samples being within 15% of their mean. Passing-Bablok regression for the difference between the systems for all analytes is depicted in Fig. 2, accompanied by Bland Altman plots, and further details are provided in Table 3. Retention times as compared to standard LC all had acceptable bias, being of –2.1% for tacrolimus, –2.0% for sirolimus, –1.9% for everolimus, –2.9% for cyclosporin A and [²H₁₂]-cyclosporin A, and –4.3% for ascomycin.

The net run-to-run time was improved from 2.3 min per injection to 0.98 min, which more than halves the time to result per sequence. Using the dual LC-MS/MS in a clinical setting with a peak intensity of 180 injections, the time until result is 3 h instead of 7 h.

3.3. Quality control

The proficiency testing program of 2019 until 2022 was followed for the four immunosuppressants, and all analyses passed the requirements that were defined by the program regulations. The long-term established measurement uncertainty over 2021 and 2022, after introduction of the Dual LC-MS/MS, was 5.3% for tacrolimus ($n = 54$), 10.3% for sirolimus ($n = 54$), 7.2% for

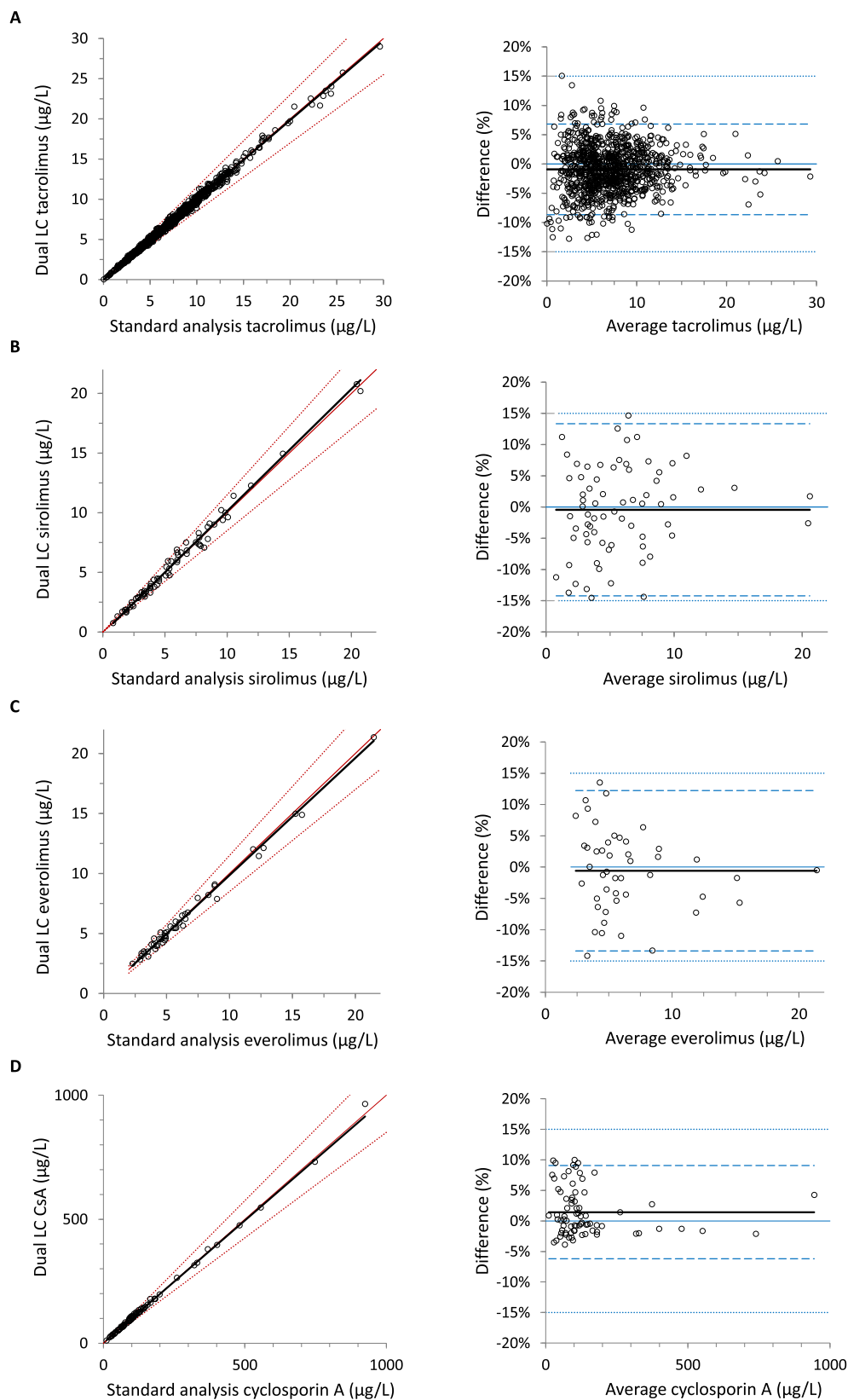


Fig. 2. Scatter plots (left) and Bland-Altman plots (right) for comparison of the concentration measurements of the standard (single) LC-MS/MS system and the dual LC-MS/MS system, with tacrolimus in 980 samples (panel A), sirolimus in 70 samples (panel B), everolimus in 46 samples (panel C), and cyclosporin A in 78 samples (panel D). On the x-axis, concentrations are shown for the analyte of interest as measured by the standard LC-MS/MS system, and on the y-axis concentrations are shown as measured by the dual LC-MS/MS system. Passing-Bablok regression fit is shown by a solid black line. The solid red line shows $x = y$ (for reference), and dotted lines represent the clinical allowable difference of $\pm 15\%$. For Bland Altman plots, mean bias is shown with a solid black line. Dashed lines show limits of agreement and the dotted lines represent the clinical allowable difference of $\pm 15\%$.

Table 3

Summary of agreement between the dual LC-MS/MS system versus the standard system, when clinical samples were measured on both systems. Slope and intercept as estimated with Passing Bablok, with 95% confidence interval (95% CI). Bland Altman mean difference with 95% CI and the 95% limits of agreement (LoA).

Dual LC-MS/MS versus standard system	Passing Bablok		Bland Altman, relative	
	Slope (95% CI)	Intercept (95% CI)	Bias,% (95% CI)	LoA,%
Analyte				
Tacrolimus (<i>n</i> = 980)	0.99 (0.99; 1.00)	−0.02 (−0.04; 0.01)	−0.93 (−1.17; −0.68)	8.67; 6.81
Sirolimus (<i>n</i> = 70)	1.02 (0.99; 1.05)	−0.11 (−0.26; 0.03)	−0.44 (−2.12; 1.23)	−14.23; 3.34
Everolimus (<i>n</i> = 46)	0.98 (0.93; 1.01)	0.09 (−0.15; 0.34)	−0.58 (−2.52; 1.36)	−13.39; 2.23
Cyclosporin A (<i>n</i> = 78)	0.99 (0.98; 1.01)	0.88 (−0.23; 2.46)	1.43 (0.55; 2.32)	−6.20; 9.07

everolimus (*n* = 27), and 8.4% for cyclosporin A (*n* = 54). For 2019 and 2020, the measurement uncertainty for the standard system was 6.4% for tacrolimus (*n* = 72), 7.7% for sirolimus (*n* = 71), 12.4% for everolimus (*n* = 36), and 7.2% for cyclosporin A (*n* = 72).

4. Discussion

The described ultra-rapid high throughput dual LC-MS/MS system configuration was developed for the therapeutic drug monitoring of tacrolimus, sirolimus, everolimus and cyclosporin A. The run-to-run time per injection was improved from 2.3 min on the standard system to a net time of 0.98 min, with consistent results. Through increasing LC-MS/MS capacity, growing numbers of injections can be handled without the need to install additional LC-MS/MS systems, which require additional room in the laboratory and have higher costs. The system is robust and has been applied for over two years without problems, and the number of drugs measured on this system is expanding.

The effort to enhance turnaround times is pursued as an asset for patient care, especially for therapeutic drug monitoring of immunosuppressants. The ideal situation would be that results from venepuncture in the morning could be discussed with the patient at the actual patient visit on the same day. While this is not possible in most laboratory flows, even results reported some hours later may reduce work overtime, as the physicians should be able to reach their patients within working hours. To this end, some novel ultrafast LC-MS/MS techniques have been described to quantify immunosuppressants in a rapid manner, including laser diode thermal desorption [16] and solid phase extraction cartridges, ranging from <15 s to 2.5 min per analysis [17,18]. In contrast, newly reported fully automated LC-MS/MS systems for immunosuppressants still last 4 min per sample [19].

In comparison, our lab made significant throughput increases that were possible by method optimisation and adding components to a standard LC-MS/MS that was already in use. Previous efforts in our lab showed already an 42% decrease in run time from 2.6 min to 1.5 min per sample by optimising the chromatography for UPLC. This was possible due to the change from a quaternary to a 1500 bar binary pump. A further 51% saving in time was gained by the change to the dual MS system configuration presented here, which mostly optimises the time lost by system reconditioning and sample loading. The reduction of net run-to-run time from 2.3 min to 0.98 min saves more than three hours on a peak day of about 180 injections, and thus leaves time for other analyses on the system. The components needed that were added to our previous system had purchase costs of 80.000 euros, which is considerably less than the costs of a new second LC-MS/MS system, estimated 300.000 euros. As no changes were made to the sample preparation, the technician time needed in the lab is the

same. Also, there were no changes to the workflow, e.g. all samples can be stored in the same autosampler, and the technicians can use the same user software.

As previously reported, there may be a rise in use of fully automated systems that are also used for immunosuppressant analysis [19]. These systems require less technician time and knowledge, but also have less flexibility to change the apparatus and methods, additional to a possible deprivation of existing knowledge in the laboratory. The current setting, a laboratory of an academic hospital, can create a challenging environment where technicians' ideas lead to the development and upgrading of site-specific systems and methods. This requires skilled technicians with extensive insights into their field of work and with a broad knowledge of state of the art techniques. This work was the result of such an environment.

The concept of dual LC-MS/MS as presented here has also been further extended to other bioanalyses performed to practice therapeutic drug monitoring in our laboratory, such as mycophenolic acid, various antipsychotic drugs with their metabolites, and the antifungals voriconazole, posaconazole, and fluconazole.

5. Conclusion

We have developed a robust dual LC-MS/MS system configuration to double the throughput for immunosuppressant therapeutic drug monitoring by maximising the utilisation of the system, and performed a Performance Qualification. With minimal reconfiguration and no changes to the workflow and the method, net run-to-run time was significantly improved in comparison to our standard LC-MS/MS system. The method is applied for clinical routine with frequent peak intensities of >180 injections per day.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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.

Data availability

Data will be made available on request.

CRediT author statement

Tanja Zijp: Formal analysis, Data curation, Writing original draft, review & editing, Visualisation. **Kai van Hateren:** Concep-

tualization, Methodology, Investigation, Validation, Writing review. **Hiltjo Kuiper:** Data Curation, Project administration, Writing review. **Erwin Jongedijk:** Conceptualisation, Methodology, Investigation, Validation, Writing review & editing. **Daan Touw:** Supervision, Conceptualization, Writing review & editing.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.chroma.2023.464086](https://doi.org/10.1016/j.chroma.2023.464086).

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