Assuring the Proper Analytical Performance of Measurement Procedures for Immunosuppressive Drug Concentrations in Clinical Practice: Recommendations of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology Immunosuppressive Drug Scientific Committee

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Abstract: Monitoring immunosuppressive drugs (ISDs) in blood or plasma is still a key therapeutic drug monitoring (TDM) application in clinical settings. Narrow target ranges and severe side effects at drug underexposure or overexposure make accurate and precise measurements a must. This overview prepared by the Immunosuppressive Drugs Scientific Committee of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology is intended to serve as a summary and guidance document describing the current state-of-the-art in the TDM of ISDs.

Key Words: TDM, immunosuppressant drugs, cyclosporine, tacrolimus, sirolimus, everolimus, mycophenolic acid, LC-MS/MS, immunoassay, design, validation, verification

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INTRODUCTION

Proper analytical test performance is a key element for successful laboratory medicine. This is particularly true when measurement platforms featuring different measurement principles are used to tackle an analytical task. Consequently, ensuring the comparability of results among laboratories and among methods has become one of the most challenging tasks in the analytical field. Method traceability, the establishment of reference methods, reference materials, and a clear concept of the measurand addressed are the cornerstones of such an undertaking and must be discussed in a global context. Conversely, individual local laboratory services, particularly if motivated to develop a "home-brew" or laboratory-developed test (LDT), need clearcut guidance and recommendations on how to design, implement, and run a routine test. Such guidance is often lacking. Too often, general validation guidelines do not meet the needs of a specific application in a routine clinical setting, which is generally characterized by high-sample throughput, the clinical need for fast turnaround, and challenging sample materials. During the past decades, different consortia and authorities, including the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT), have published several overviews for therapeutic drug monitoring (TDM) of immunosuppressant drugs (ISDs).[1–14] Although these documents are highly recommended for summarizing the available pharmacokinetic(PK)/pharmacodynamic (PD) and TDM test performance data of the ISDs, such as cyclosporine, tacrolimus, sirolimus, everolimus, and mycophenolic acid, they do not specifically address the design and execution of validation and/or verification protocols that demonstrate the fitness of a measurement procedure for its intended purpose, such as the analysis of immunosuppressive drugs, in clinical samples to guide therapy. In addition, there is an ongoing need to update these documents to reflect current clinical needs and technical achievements.

PHARMACOKINETIC TDM OF ISDs

According to the definition provided by the IATDMCT,[15] TDM is a multidisciplinary clinical specialty aimed at improving patient care by individually adjusting the dose of a drug for which clinical experience or clinical trials have shown that such an adjustment improved the outcome in a general or specific population. Along with demographic, clinical, pharmacogenetic, and PD data, measurement of blood concentrations of drugs at known sampling times (PK monitoring) is a central part of TDM. In general, TDM is warranted whenever the dose–effect relationship does not allow a "one size fits all" drug administration policy.[16,17]

ISD therapy requires close monitoring of drug concentrations because of several reasons:

1. The anticipated target range of these drugs (usually measured as the trough concentration) is narrow.
2. Consequences are severe if the target range is missed—drug toxicity and/or overimmunosuppression resulting in excessive risk for infection and malignancies can occur; conversely, graft function impairment and graft loss are possible.
3. Toxicodynamic effects can be difficult to distinguish from clinical disease (eg, nephrotoxicity caused by calcineurin inhibitors from impairment of kidney graft function or from BK virus nephropathy).
4. The dose/exposure relationship is interindividually and intraindividually highly variable. Dose and target range adjustment is patient specific and dose/concentration relationships are complicated by a variety of confounding factors, including, but not limited to, genetic polymorphisms, drug–drug, drug–disease, drug–food, and drug–environment interactions.
5. Adherence to ISD regimens is critical and requires closer monitoring, especially in adolescents and in pediatric patients.

ANALYTICAL MEASUREMENT FRAMEWORK

Responsible state-of-the-art TDM can only be based on a reliable measurement system—"TDM assays."[18] The key to a reliable and valid TDM assay is adequate method design and thorough method validation, a sound and well-introduced scientific concept to demonstrate the fitness of a method in the framework of its anticipated use.[19] A variety of different guidelines are available to design experiments leading to properly validated analytical methods. Such guidelines are often rather general. Representative examples are the International Collaborative Exercise (ICE) guideline "Validation of analytical procedures,"[20] the "The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics"[21] guideline issued by Eurachem or the latest industry-related Food and Drug Administration (FDA) guidance document, "Analytical procedures and methods validation for drugs and biologics".[22] Others are more specific, such as the European Medicines Agency (EMEA) "Guideline on bioanalytical method validation"[23] and the FDA "Guidance for industry—bioanalytical method validation"[24] that focus on the application of analytical methods in the pharmaceutical industry and are useful for drug development, particularly for bioequivalence/PK studies. Nevertheless, additional, more specific recommendations for the clinical analysis of drugs that have their own individual challenges, such as ISDs, are required.

Recommendations that come closest to assay application in a clinical laboratory setting are summarized in several guidance documents issued by Clinical and Laboratory Standards Institute (CLSI),[25] but they also do not address all the specifics of a TDM assay operation (design, validation, and life-cycle management), neither for chromatographic platforms nor for ligand-binding assays. In fact, some characteristic issues must be considered when implementing TDM services for ISDs (Table 1). These include, but are not limited to, the validity of the target range established with different methods, adequacy of the measurement range with steadily changing therapeutic schemes and developing of TDM strategies, accounting for drug metabolites, coadministered drugs, and method consistency over time that impacts the long-term clinical outcome.

It is the responsibility of an individual TDM provider to determine the appropriate guidance framework, which the quality management system will be based on (eg, ISO 15189, ISO 17025, ISO 9001, or CAP and/or the CLSI document family). In this context, one challenge to consider is that TDM assay performance has to be controlled and consistent in the long-term in the context of changing personnel, limited long-term availability of hardware/software components, and reagents. Hence, evaluating robustness of the assay and a risk analysis, (eg, using the failure mode and effect analysis) approach as outlined in ISO 14971, should be conducted along with the development process to identify, minimize, and communicate possible risks for key assay elements. Generally regulatory framework guidelines published by both international scientific societies and governmental agencies can be adopted for the analysis of ISDs when considering specifics related to their TDM.

The second major pillar of analytical science is the proper use of analytical equipment. Similar to analytical methods that must be validated, analytical instruments must be qualified.[44] Their use cycle starts with a design phase ensuring that the right instrument is chosen for the intended purpose, followed by the proper installation of the equipment (installation qualification/operational qualification). Once qualified, such an instrument can be used as the technological basis for analytical assay development and validation. Before being used by an analytical service as TDM measurements in routine clinical practice, a performance qualification (eg, a performance evaluation study) that demonstrates the long-term stability and performance of an instrument/assay combination under typical routine and extreme conditions must be performed.

Throughout the life-cycle of a TDM service, it is important to implement a system for verification/revalidation so that the analytical specifications of an established measurement procedure are achieved and continuously maintained.

Taken together, it is the responsibility of the individual laboratory to put together a tailored set of standard operating procedures for the design, development, validation,

TABLE 1. Drug Specific Considerations to Support Assay Development for Immunosuppressive Drugs

Drug	Distribution in Blood	TDM Sample Matrix	Monitored PK Parameter	Typical Concentrations in ISD-TDM Samples	Major Metabolites			Stability in Sample Matrix*	References
					Name	Bioactivity (% of Parent Drug)	Concentration Relative to Parent Drug (% at t0)		
Cyclosporine	41%–58% in erythrocytes	Whole blood	C0	50–350 mcg/L	AM1 (1-β-Hydroxyl)	10–20	80–150	AT: 7 d	8,25–31
			C2	480–2000 mcg/L	AM 9 (9-γ-hydroxy)	5–10	50–75	2–8°C: 7 d	
					AM 4N (4-N-desmethyl)	3–5	5–25	−20°C: 3 yrs	
Tacrolimus	~85% in erythrocytes	Whole blood	C0	3–15 mcg/L	M I (13-O-desmethyl)	6	6.4	AT (22°C): 14 d	9,11,27,30,32–35
					M II (31-O-desmethyl)	100	ND	2–8°C: 14 d	
					M III (15-O-desmethyl)	0	5.3	−70°C: 1 yr	
					M IV (12-hydroxy)	3.5	ND		
					M V (15, 31-O-didesmethyl)	0	ND		
					M VII (13, 15-O-didesmethyl)	0	1.7		
Sirolimus	~95% in erythrocytes	Whole blood	C0	3–20 mcg/L	39-O-desmethyl	10	5	AT (30°C): 8 d	6,14,30,36–38
					16-O-desmethyl	ND	8.6	4°C: 30 d	
					12-hydroxy	7	11	−40°C: 2 mo	
					27, 39-O-didesmethyl	ND	31		
Everolimus	>75% in erythrocytes	Whole blood	C0	3–15 mcg/L	46-hydroxy	ND	44	AT (30°C): 7 d	30,39–41
					24-hydroxy	ND	7.7	2–8°C: 7 d	
					25-hydroxy	ND	14.4	−80°C: 2 yrs	
Mycophenolic acid†	~99.9% in plasma	Plasma (preferentially EDTA plasma)	C0	1–4 mg/L	Mycophenolic acid glucuronide	No bioactivity	20–100 fold higher	AT: 8 h	10,13,42,43
			Abbreviated AUC (LSS)	30–60 $mg \cdot h^{-1} \cdot L^{-1}$ (Cmax ~10–55 mg/L)	Mycophenolic acid acyl glucuronide	Minor	4.6%–45.5%	2–8°C: 4 d −20°C: 11 mo	

*As reported in the literature, no extreme ambient temperatures evaluated. Transport and storage of whole blood clinical and clinical trial samples at ambient temperature for more than 3 days is discouraged due to the increasing risk of matrix degradation. Refrigeration at +4°C within 1 day of collection and freezing after 1 week is recommended.

†Active moiety of mycophenolate mofetil and mycophenolate sodium.

AT, ambient temperature; AUC, area under the concentration–time curve; C0, predose (trough) concentration; C2, concentration 2 hours after drug intake; Cmax: maximum concentration; EDTA, Ethylenediaminetetraacetic acid; ISD, immunosuppressive drug; LSS, limited sampling strategies; ND, not determined; PK, pharmacokinetic; t0, predose sampling time; TDM, therapeutic drug monitoring.

deployment, and life-cycle management of a testing procedure within the framework of international or national state-of-the-art regulations and procedures and local regulatory requirements. In addition (and this goes far beyond the responsibility of the individual laboratory), it is in general highly desirable to achieve a high level of intralaboratory and interlaboratory standardization of sample workflows and analytical measurements to minimize intralaboratory variability and interlaboratory bias, which can manifest as interlaboratory variability that typically markedly exceeds intralaboratory variability.

Whereas, as stated above, intralaboratory precision enhancement is in the hands of the individual laboratory, interlaboratory bias can only be addressed by an initiative focusing on the establishment of higher-order reference materials and/or higher-order reference procedures to ensure worldwide measurement traceability.[45–47] Such initiatives have been an integrative part of laboratory medicine for decades; numerous higher-order reference procedures and reference materials are available for key diagnostic parameters, for example, glucose,[48] creatinine,[49] hemoglobin A1c,[50] enzymes,[51,52] and hormones.[53] For a complete listing of reference materials, reference methods and reference measurement services available, see the Web site of the Joint Committee for Traceability in Laboratory Medicine (www.bipm.org/jctlm, JCTLM). Consortia have undertaken standardization projects for different endocrinological parameters, such as testosterone,[54] estradiol,[55] thyroid hormones,[56] 25-OH-vitamin D,[57] human growth hormone and insulin-like growth factor 1,[58] but such initiatives are still lacking for most TDM-relevant drugs, including ISDs. Currently, only one ISD reference material—a commercially available whole-blood standard for tacrolimus (ERM-DA110a)—is listed in the JCTLM database. Some candidate reference methods have been published[59]; however, none has been reviewed and listed by the JCTLM. Consequently no reference measurement service is available to ensure the comparability of routine platforms through the establishment of a traceability chain.

We are not in the position to provide validation and qualification guideline information in this report, but we intend to highlight analytical issues that need to be addressed when properly designing and validating an ISD-TDM assay, taking into account the diagnostic requirements in a routine clinical setting.

CURRENT LABORATORY PRACTICES FOR THE TDM OF ISDs

In 2013 under the auspices of the IATDMCT, a structured web-based survey was conducted to systematically document and analyze current practices for the TDM of ISDs in clinical laboratories and to identify potential causes of the substantial interlaboratory variability observed in proficiency-testing programs.[60] This comprehensive survey consisted of 128 questions organized in 8 sections that covered assay development and the entire assay testing process, from sample acquisition to the reporting of the results, the regulatory laboratory environment and personnel qualification and training. Seventy-six laboratories in 14 countries completed the survey.

Cyclosporine was the only ISD for which immunoassays were primarily used for TDM (53% versus 47% LC-MS/MS, n = 76). For TDM of tacrolimus, 53% of the laboratories used LC-MS/MS and 47% used immunoassays (n = 72). The inhibitors of the mammalian target of rapamycin (mTOR), such as sirolimus and everolimus, were quantified predominantly by LC-MS/MS (sirolimus: 70% LC-MS/MS, 29% immunoassay, 1% high performance liquid chromatography with ultraviolet light detector, n = 66 and everolimus: 75% LC-MS/MS, 23% immunoassay, 2% HPLC/UV, n = 51). In the TDM of mycophenolic acid, 26% of the laboratories used immunoassays, 37% used LC-MS/MS and 37% used HPLC/UV (n = 52). Fifty-nine percent of the LC-MS/MS assays (n = 37) were completely developed in house, whereas the others were based on commercial kits. More than 75% of the LC-MS/MS assays were multianalyte assays that could simultaneously quantify cyclosporine, tacrolimus, sirolimus, and everolimus. Table 2 summarizes the currently available analytical methods for the TDM of ISDs.

Based on the survey results, it can be summarized that interlaboratory variability of TDM for ISDs is a multifactorial problem that includes all the aspects of the laboratory operation, such as the analytical assays, the quality system, and the training policy. The major findings included, but were not limited to that mentioned in the following sections.

Sample Handling, Shipping, and Storage

Laboratories received samples from local, regional, and national sources and the times from sample collection to analysis varied from a couple of hours to 1 week. Many laboratories did not control the temperature during this time and 15% of the laboratories did not seem to have specific procedures for temperature control of samples during transport and storage at all. A large percentage of laboratories did not reject partially clotting whole-blood samples (29%), or those collected using an incorrect anticoagulant during sample acquisition (43%), or those that did not meet requirements for a minimal sample volume (53%). Sample preparation was mostly manual (72%). The extraction procedures were often poorly controlled and standardized.

LC-MS/MS Assays

Although isotope-labeled internal standards are considered to be state-of-the-art for LC-MS/MS assays, 62% of the laboratories used ascomycin as the internal standard for tacrolimus analysis and, in some cases, also for the quantification of structurally less-related compounds, such as sirolimus (29%) and cyclosporine (6%). There was marked variability in the storage conditions and expiration dates of reference material stock solutions.

Calibrators and QCs

Stock solutions for the preparation of calibrators were also used for QC samples by 34% of the laboratories. This is a potential source of error unless the accuracy of the common stock solution is verified and does not rely solely on the weight of the reference material alone. In addition, 25% of the laboratories used serial dilutions for the preparation of their calibrators.

TABLE 2. Analytical Performance of Currently Available Methods as Reported by the Manufacturers

Drug	Method	Manufacturer	Analytical Platform	Measurement Range*	Total Method Imprecision Over the Therapeutic Concentration Range	Inaccuracy, (Method Comparison with a Validated Reference method†)	Specificity for the Parent Drug (% Crossreactivity with Metabolites and Structurally Related Drugs)	References
Cyclosporine	LC-MS/MS‡	Different commercial or lab developed tests	Various LC-MS/MS platforms	10–2000 mcg/L	≤10%, over the measurement range	Typically serves as the reference method	Specific for the parent drug (AM1, AM9 and AM19 are separated from the analyte)	61–64
	EMIT 2000	Siemens Healthcare Diagnostics	General chemistry analyzers using spectrophotometric detection	40–500 mcg/L, 350–2000 mcg/L (CSA extended range)	<20%, at concentrations ≥72 mcg/L	Clinical samples, (LTx, HTx, and KTx, n = 136), NS; intercept: −3 to 24, slope: 0.92 to 1.14, r = 0.828 to 0.975	AM1: −5.9% to <0.3%, AM1c: 1% to 2.7%, AM4N: <0.3% to 0.9%, AM9: 5.5% to 7.3%, AM19: −2.7% to 3.0%, AM1c9: −2.5% to 1.9%, AM4N9: 1.6% to 2.0%	65
						CSA extended range, clinical samples (LTx, HTx, and KTx, n = 138), PB; intercept: −49.0 to 19.0, slope: 1.00 to 1.14, r = 0.97151 to 0.989		
	CEDIA PLUS	Thermo Scientific	General chemistry analyzers using spectrophotometric detection	Low assay: 40–450 mcg/L, High assay: 450–2000 mcg/L	≤16% at concentrations ≥46 mcg/L	CSA High assay, clinical samples (LTx, HTx, and KTx, n = 311), DE; intercept: 9 to 23, slope: 0.93 to 1.18, r = 0.91 to 0.94	AM1: 4.4%; AM1c: 1.6%; AM4N: 16%; AM9: 20%; AM19: 0.9%; AM4N9: 1.0%	66
						CSA High assay, clinical samples (LTx and KTx, n = 93) DE; intercept: 70 to 84, slope: 0.98 to 1.02, r = 0.96 to 0.97		
	ACMIA	Siemens Healthcare Diagnostics	Dimension and Dimension Vista clinical chemistry systems	30–500 mcg/L, 350–2000 mcg/L (CSA extended range)	<9% at concentrations ≥65 mcg/L	Clinical samples (LTx, HTx, and KTx, n = 661) PB; intercept: −8.9 to −16.0, slope: 1.13 to 1.28, r = 0.893 to 0.961	AM1: 1.8% to 4.7%; AM1c: 1.1% to 2.1%; AM4N: 3.1% to 6.0%; AM9: 2.1% to 2.4%; AM19: 1.8% to 3.0%; AM1c9: 1.7% to 3.9%	67,68
						CSA extended range, clinical samples (LTx, HTx, and KTx, n = 140) PB; intercept: 15.2 to 93.7, slope: 0.93 to 1.10, r = 0.980 to 0.990		
	CMIA	Abbot Laboratories	Architect *i*	30–1500 mcg/L	≤15% more than the measurement range	Clinical samples (LTx, HTx, and KTx, n = 227) PB; intercept (95% CI): −24.94 (−32.71 to −19.18), slope (95% CI): 1.20 (1.17 to 1.23), r = 0.990	AM1: −0.7% to 1.7%; AM1c: −0.8% to 3.3%; AM4N: −2.3% to 3.1%; AM9: −3.8% to 1.9%; AM19: −2.9% to 2.1%	69

TABLE 2. (*Continued*) Analytical Performance of Currently Available Methods as Reported by the Manufacturers

Drug	Method	Manufacturer	Analytical Platform	Measurement Range*	Total Method Imprecision Over the Therapeutic Concentration Range	Inaccuracy, (Method Comparison with a Validated Reference method†)	Specificity for the Parent Drug (% Crossreactivity with Metabolites and Structurally Related Drugs)	References
	ADVIA Centaur	Siemens Healthcare Diagnostics	ADVIA Centaur, ADVIA Centaur XP, and ADVIA Centaur XPT systems	30–1500 mcg/L	≤9% at concentrations ≥84 mcg/L	Clinical samples (LTx, HTx and KTx, n = 250) DE; intercept: −15.0 to 35.0, slope: 0.88 to 1.14, r = 0.909 to 0.978	AM1: <5%; AM1c: <5%; AM4N: <5%; AM9: 15%; AM19: <5%	70
	ECLIA	Roche Diagnostics	Elecsys 2010, Modular Analytics E170, cobas e 411, cobas e 601, cobas e 602	50–2000 mcg/L	<10% at concentrations ≥63 mcg/L	Clinical samples (n = 352) PB; intercept: 2.08, slope: 1.09, r = 0.900	AM1: 2%; AM1c: n.d.; AM4N: 2%; AM9: 6%; AM19: n.d.; AM1c9: n.d.	71
Tacrolimus	LC-MS/MS‡	Different commercial or lab developed tests	Various LC-MS/MS platforms	0.5–50 mcg/L	≤10% over the measurement range	Typically serves as the reference method	Specific for the parent drug	62–64
	EMIT 2000	Siemens Healthcare Diagnostics	General chemistry analyzers utilizing spectrophotometric detection	2.8–30 mcg/L	<17% at concentrations ≥5.1 mcg/L	Clinical samples (LTx and KTx, n = 155) NS; intercept: −0.12 to 0.35 slope: 1.01 to 1.10, r = 0.899 to 0.975	M I: 10.4% (13-*O*-desmethyl-); M II: 1.6% (31-*O*-desmethyl-); M III: 2.2% (15-*O*-desmethyl-); M IV: 21.1% (12-hydroxy-); M V: 2.5% (15, 31-*O*-didesmethyl-); M VI: 0.6% (13, 31-didesmethyl-tacrolimus); M VII: 0.9% (13, 15-O-didesmethyl-tacrolimus); M VIII: 2.2%	72
	ACMIA	Siemens Healthcare Diagnostics	Dimension clinical chemistry systems	1.0–30 mcg/L	<9% at concentrations ≥1.8 mcg/L	Clinical samples (LTx and KTx, n = 201) PB; intercept (95% CI): −0.26 (−0.42 to 0.0), slope (95% CI): 1.04 (1.00 to 1.07), r = 0.98	M I: 1.0% (13-*O*-desmethyl-); M II: 18.0% (31-*O*-desmethyl-); M III: 15.0% (15-*O*-desmethyl-); M IV: 99% (12-hydroxy-); M V: 1.0% (15, 31-*O*-didesmethyl-); M VI: 1.0% (13, 31-O-didesmethyl-tacrolimus); M VII: 43.0% (13, 15-O-didesmethyl-tacrolimus); M VIII: n.d.	73
	CMIA	Abbot Laboratories	Architect *i*	2.0–30 mcg/L	≤10% over the measurement range	Clinical samples (n = 125) PB; intercept (95% CI): 0.22 (0.02 to 0.48), slope (95% CI): 1.07 (1.01 to 1.12), r = 0.92	M I: 8% (13-*O*-desmethyl-); M II: 94% (31-*O*-desmethyl-); M III: 45% (15-*O*-desmethyl-); M IV: 9% (12-hydroxy-)	74
	ECLIA	Roche Diagnostics	Elecsys 2010, Modular Analytics E170, cobas e 411, cobas e 601, cobas e 602	1.0–40 mcg/L	<15% at concentrations ≥1.3 mcg/L	Clinical samples (n = 206) PB; intercept: −0.184 slope: 1.052, r = 0.93	M I: n.d. (13-*O*-desmethyl-); M II: 70% (31-*O*-desmethyl-); M III: n.d. (15-*O*-desmethyl-); M IV: n.d. (12-hydroxy-)	75

(*continued on next page*)

TABLE 2. (*Continued*) Analytical Performance of Currently Available Methods as Reported by the Manufacturers

Drug	Method	Manufacturer	Analytical Platform	Measurement Range*	Total Method Imprecision Over the Therapeutic Concentration Range	Inaccuracy, (Method Comparison with a Validated Reference method†)	Specificity for the Parent Drug (% Crossreactivity with Metabolites and Structurally Related Drugs)	References
	QMS	Thermo Scientific	General chemistry analyzers utilizing spectrophotometric detection	1.0–30 mcg/L	<8% at concentrations ≥3.0 mcg/L	Clinical samples (LTx, HTx and KTx, n = 232) DE; intercept (95% CI): 0.71 (0.42 to 1.01), slope (95% CI): 1.13 (1.092 to 1.167), r = 0.967	M I: 7.7% to 9.2% (13-*O*-desmethyl-); M II: −0.5% to 0.7% (31-*O*-desmethyl-); M III: 2.7% to 3.8% (15-*O*-desmethyl-); M IV: 5.7% to 174.8% (12-hydroxy-); M VII 6.7% to 9.3% (13,15-O-didesmethyl-)	76
Sirolimus	LC-MS/MS‡	Different commercial or lab developed tests	Various LC-MS/MS platforms	0.5–50 mcg/L	≤10% over the measurement range	Typically serves as the reference method	Specific for the parent drug	62–64
	CMIA	Abbot Laboratories	Architect *i*	2.0–30 mcg/L	≤10% over the measurement range	Clinical samples (KTx, n = 168) PB; intercept (95% CI): −0.37 (−0.89 to 0.12), slope (95% CI): 1.18 (1.11 to 1.27), r = 0.91	39-*O*–desmethyl; 20.3%; 6-*O*-desmethyl: 7.6%; 12-hydroxy: 36.8%; 41-*O*-desmethyl-hydroxy: 8.7%	77
	ACMIA	Siemens Healthcare Diagnostics	Dimension clinical chemistry systems	2.0–30 mcg/L	<15% at concentrations ≥2.7 mcg/L	Clinical samples (n = 119, KTx = 83) NS; intercept (95% CI): −0.7 (−1.26 to 0.06), slope (95% CI): 1.20 (1.13 to 1.27), r = 0.95	39-O-desmethyl: 52% to 60%; 16-O-demethyl: 6% to 10%; 12-hydroxy: 46% to 53%; 27, 39-O-didesmethyl: 83% to 89%	78
	EMIT 2000	Siemens Healthcare Diagnostics	General chemistry analyzers utilizing spectrophotometric detection	3.5–30 mcg/L	<14% at concentrations ≥5.0 mcg/L	Clinical samples (KTx, n = 128) NS; intercept: 0.054, slope: 1.30, r = 0.946	39-O-desmethyl: 38% to 42%; 16-O-desmethyl: 15% to 21%; 12-hydroxy: 4% to 6%; 27, 39-O-didesmethyl: 52% to 61%	79
Everolimus	LC-MS/MS‡	Different commercial or lab developed tests	Various LC-MS/MS platforms	0.5–50 mcg/L	≤10% over the measurement range	Typically serves as the reference method	Specific for the parent drug	62–64
	QMS	Thermo Scientific	General chemistry analyzers utilizing spectrophotometric detection	1.5–20 mcg/L	<10% at concentrations ≥3.9 mcg/L	Clinical samples (KTx, n = 150; HTx, n = 41; LTx, n = 111) PB; intercept: KTx: −0.005; HTx: −0.15; LTx: 0.98; slope: KTx: 1.11; HTx: 1.00; LTx: 0.98, r = 0.93 to 0.96	40-phosphatidylcholine-: 59% to 63%; 45,46-dihydroxy -: ≤2%; 24-hydroxy-: ≤9%; 25-hydroxy-: ≤22%; ring-open compounds (seco acid and precursor seco acid of everolimus): 2% to 16%; sirolimus: 46%; 11-hydroxy-sirolimus: 12%; 41-*O*-desmethyl-; 32-O-desmethyl-sirolimus: 45%; other sirolimus metabolites: ≤7%	80

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Drug	Method	Manufacturer	Analytical Platform	Measurement Range*	Total Method Imprecision Over the Therapeutic Concentration Range	Inaccuracy, (Method Comparison with a Validated Reference method†)	Specificity for the Parent Drug (% Crossreactivity with Metabolites and Structurally Related Drugs)	References
Mycophenolic Acid	LC-MS/MS or HPLC/UV‡	Different commercial or lab developed tests	Various LC-MS/MS or HPLC/UV (DAD) platforms	0.1–50 mg/L	≤10% over the measurement range	Typically serves as the reference method	Specific for the parent drug (mycophenolic acid glucuronides are separated)	62–64,81
	EMIT 2000	Siemens Healthcare Diagnostics	General chemistry analyzers utilizing spectrophotometric detection	0.1–15 mg/L	<7% at concentrations ≥1 mg/L	Clinical samples (n = 155) DE; intercept: 0.377, slope: 1.04, r = 0.987	Mycophenolic acid glucuronide: n.d.; Mycophenolic acid acyl glucuronide: 10% to 30%; Mycophenolate mofetil: 64%	82
	IMPDH-based enzyme inhibition assay	Roche Diagnostics	cobas c 311, cobas c 501/502, cobas Integra 400 plus, cobas Integra 800	0.4–15 mg/L	<4% at concentrations ≥0.85 mg/L	Clinical samples (KTx, n = 88; HTx, n = 70) PB; intercept (95% CI): 0.016 (−0.029 to 0.057), slope (95% CI): 1.062 (1.043 to 1.084), r = 0.99 (performed on cobas c 501)	Mycophenolic acid glucuronide: n.d.; Mycophenolic acid acyl glucuronide: 6.5%	83
	CEDIA	Thermo Scientific	General chemistry analyzers using spectrophotometric detection	0.3–10 mg/L	<10% at concentrations ≥1 mg/L	Clinical samples (KTx, n = 92; HTx, n = 96) DE; intercept (95% CI): 0.12 (−0.01 to 0.25), slope (95% CI): 1.089 (1.051 to 1.128), r = 0.970	Mycophenolic acid glucuronide: n.d.; Mycophenolic acid acyl glucuronide: 133.3% to 177.8%	84
	PETINIA	Siemens Healthcare Diagnostics	Dimension clinical chemistry systems	0.1–30 mg/L	<7% at concentrations ≥1 mg/L	Clinical samples (KTx, n = 115; HTx, n = 86; LTx, n = 64) NS; intercept: 0.17 to 0.28, slope: 1.08 to 1.18, r = 0.985 to 0.995	Mycophenolic acid glucuronide: 0.6%; Mycophenolic acid acyl glucuronide: 36.8% to 64.5%; Mycophenolate mofetil: 28.6% to 30.5%	85

*The lower limit of the measurement range is defined either by the limit of quantification (LOQ) or by the "functional sensitivity" respective to what is reported by the manufacturer.

†Validated reference method = LC-MS/MS except MPA (=HPLC), data shown are representative examples that may vary between patient populations for example with different type of transplantation; Regression model uses: PB = Passing Bablok, DE = Deming, NS = not stated.

‡Performance characteristic for state-of-the-art LC-MS/MS methods.

ACMIA, antibody conjugated magnetic immunoassay; CEDIA, cloned enzyme donor immunoassay; CMIA, chemiluminescent microparticle immunoassay; CSA, cyclosporine; ECLIA, electrochemiluminescence immunoassay; EMIT, enzyme multiplied immunoassay technique; HPLC-UV, high-pressure liquid chromatography with ultraviolet detection; HTx, heart transplantation; KTx, kidney transplantation; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LTx, liver transplantation; n.d., not detectable; PETINIA, particle enhanced turbidimetric inhibition immunoassay; QMS, Quantitative Microsphere System.

Assay Validation, Long-Term Performance Tracking, and Proficiency Testing

Laboratories using commercially purchased kits, immunoassay kits, and LC-MS/MS kits were less likely to validate their assay than laboratories using assays developed in-house. Among the LC-MS/MS laboratories using commercial kits, only 28% of them had assessed matrix effects (eg, ion suppression), 57% had assessed carryover, and 20% had assessed autosampler stability using their specific equipment and personnel. Eighty-nine percent of the in-house LC-MS/MS assays had been tested for matrix effects, 80% had been tested for carryover and 67% had been tested for autosampler/extracted sample stability.

Twenty percent of the immunoassays and 9% of the LC-MS/MS assays had never been fully validated in house. Once established, between 35% (LC-MS/MS) and 45% (immunoassay) of the laboratories had not revalidated their assay since their implementation. Sixteen percent of the laboratories did not have long-term assay performance tracking procedures in place and did not perform regular QCaudits. Depending on the immunosuppressant, between 14% and 19% of the laboratories did not seem to participate in any type of proficiency-testing program and had not crossvalidated their results with other laboratories. Seven percent of the laboratories did not have any of their analytical equipment on a preventive maintenance schedule.

Personnel Training and Retraining

On average, 65% of the individuals performing immunosuppressant TDM assays in the laboratory had less than 1 year of experience and, of these, 20% had less than 3 months

Only 4% of the laboratories had monthly training sessions, and 71% retrained personnel only "as necessary" and/or in response to a corrective action request. In 4% of the laboratories, there was no training policy in place. Instead of proactively maintaining an adequate educational and training level, most laboratories only retrained their personnel "after the fact," once problems had occurred.

Taken together, 3 main reasons for variability were suggested from the survey results; a lack of standardization of analytical methods and sample testing practices; a lack of the use of appropriate reference materials, calibrators, and control samples; and inconsistency of regulatory requirements and the level of compliance to internationally accepted laboratory practice guidelines in different parts of the world.

ROLE OF PROFICIENCY-TESTING DATA

Proficiency-testing data are a rich source of information. In addition to interlaboratory precision and bias,[86] average intralaboratory precision and bias can be assessed by resending identical samples at different times.[87] Addition of spiked samples with drug metabolites or the frequent inclusion of patient samples in the sample pool enables the discrimination between matrix-induced method bias ("lacking sample commutability") and crossreactivity-induced method bias. This bias will most likely be present if patient materials[88] or metabolite-fortified samples[89] are measured with different assay systems. Interlaboratory variability data are combined from an intralaboratory proportion (usually 70%–90%)[89] and from other interlaboratory contributions. Hence, if the interlaboratory imprecision is significantly higher than the intralaboratory imprecision for a specific method (eg, 20% versus 5%), it cannot be solely explained by the latter. Additional error components, most likely a constant measurement bias between at least some of the participating laboratories, are present. Such a bias can degrade a method that performs well within many individual laboratories to a method that is unfit to follow patients when testing is performed by different laboratories. In at least one case (25-OH-Vitamin D), close inspection of the data followed by experimental intervention allowed the identification of a calibration bias as source of this error.[90] LDT systems, which currently comprise mostly LC-MS/MS applications, are particularly susceptible to such sources of error. Only stringent method design and validation in a qualified environment can prevent such problematic situations.

In the UK-based proficiency-testing scheme for immunosuppressives[91] serving several hundred participating laboratories, a between-laboratory variability of $<15\%$ was observed at target (therapeutic) concentrations of tacrolimus and cyclosporine and $<20\%$ at off-target concentrations. For mycophenolic acid, sirolimus, and everolimus, the coefficient of variation (CV %) for concentrations within and below the target ranges evaluated were $<20\%$ and $<30\%$, respectively. Whereas currently available immunoassays demonstrate comparable or even better between-laboratory variability in the upper half of the ISD target range, the CVs at lower concentrations are higher and frequently exceed 20% (Fig. 1). The bias of the results reported for different immunoassays compared with that of LC-MS/MS procedures varies widely (Fig. 2). This is true both for pooled patient samples and for materials spiked with the appropriate drug, revealing that both matrix-related factors (eg, crossreactivity and interference) and inconsistency of calibration are causative. Furthermore, the negative deviation from the target value that is continually observed with some immunoassays when using spiked QC samples suggests that calibration of these assays has been adjusted to compensate for the effects of matrix factors (eg, crossreactivity with drug metabolites). For example, the method description in the assay package insert for the everolimus Quantitative Microsphere System (QMS) immunoassay[92] indicates that its calibration strategy is based on value-assigned calibrators and QC samples. According to the manufacturer, this approach was designed to align the results for samples from patients with the "average" measurements obtained by LC-MS/MS methods. However, one should be aware that the bias for individual patient samples "may vary with both direction depending on crossreactivity with metabolites and other potential errors." Moreover, as recently shown for the sirolimus microparticle enzyme immunoassay (MEIA) method, calibration bias and between-method bias may change with time which potentially could have a dangerous impact on established clinical practices.[93]

GENERAL RECOMMENDATIONS FOR THE ESTABLISHMENT OF METHODS TO MEASURE IMMUNOSUPPRESSIVE DRUGS

The life cycle of a measurement procedure can be divided into distinct phases. If a clinical need can be defined (eg, to keep a patient within a recommended drug target range), analytical goals can be formulated (eg, assay range, requirements for analytical precision, etc.). Based on such quality requirements, a rational measurement platform and method selection leads to the development or establishment of a method. Once a stable method is available, performance goals are transformed to a list of validation benchmarks that are tested by appropriate measurements. Data derived from these measurements are compared against the predefined performance goals. If all goals are met, the implementation of the measurement procedure can be undertaken. If not, the method design has failed and must be modified. Once implemented, an assay must be monitored by appropriate QC procedures (eg, system suitability testing,[94] internal QC, periodical reevaluation of test performance, proficiency testing, etc.). Whenever possible, the measurement platform life cycle should be accompanied by a risk assessment that has been initiated in the design phase of the project.

Method Design

Meaningful method design must meet clinical needs. The analysis frequency, time to report results, laboratory workflow issues, desired analytical range, and minimal requirements for assay precision (ie, derived from biological variation and the impact on clinical decision-making) are the cornerstones for measurement method selection. For ISDs, this means that a total turnaround time of 3–6 hours is desirable in a transplantation center setting to allow for daily dose adjustment in acute situations. In particular, for LC-MS/MS platform-based analytical services, this can be challenging. If only outpatient units or external care providers have to be serviced or tied to the reported results, overnight reporting can be sufficient. Characteristics specific to individual ISDs that should be considered when designing an analytical method are given in Table 1. Frequently used comedications such as other ISDs, antibiotics, antifungals, antivirals, antidiabetics, antihypertensive drugs, lipid-lowering agents, and nutritional supplements can be extracted from the respective prescribing information.

Method Validation

For In Vitro Diagnostics (IVD)-CE certified or FDA cleared commercial tests, the producer must clearly state which guideline was followed for method validation. For a LDT, this documentation must also be given. Furthermore, it must not be forgotten that hardly any of the guidelines referred to above allow complete assay validation for clinical use. Therefore, a validation plan needs to be internally developed and should consider national regulations, the type of methodology, the targeted immunosuppressive drug, its physicochemical properties and specific clinical requirements. The written validations plan must be clearly defined as such; the validation experiments need to be described in sufficient detail and predefined acceptance criteria should be stated. For an overview of key validation elements deemed necessary to characterize a bioanalytical method, the reader is referred to the literature[95–98] and to the aforementioned guidelines. Specific additional recommendations for ISD TDM methods are summarized in the following sections.

Method validation can be either a full validation or a partial validation. A full assay validation should always be performed prior to the clinical application of any newly developed method and of any method setup based on literature data. The primary performance characteristics essential to demonstrate the reliability of a method include the measurement range (including the lower [LLOQ] and upper limit of quantification [ULOQ]), assay precision and accuracy, specificity for the parent drug, robustness against interference and pitfalls specific to the chosen technique, carryover, and stability of the analyte, the sample extracts and reagents under storage and processing conditions. If the storage conditions for assay calibrators and controls (frozen) differ from those of the patient samples (fresh), investigation of fresh and thawed aliquots of identical specimens to control for calibration bias due to matrix discrepancies between thawed calibrators or controls and fresh patient samples should be part of the validation protocol as well. Moreover, if applicable, issues such as the suitability of lyophilized QC materials and calibrators used in an analytical procedure, the effects of collection tube separating gels, risk of nonspecific adsorption to surface materials (eg, glass, PE, PVC, etc.) or the use of different suppliers or different lots of reagents and other materials must be addressed during validation. When minor changes have been made to an assay (as transfer to another instrument of the same model, a change in equipment and reagent provider or a change in storage conditions, etc) a partial validation is necessary. This may also be required if a method has been out of control as for example indicated by failing long-term quality tracking acceptance criteria or proficiency testing, and if a previously validated assay has not been used for an extended period. The scope and extent of a partial validation depends on the modifications and issues requiring revalidation and may vary from evaluating a single performance characteristic to an almost full validation.[23,99]

Method Verification

Even when IVD-CE–certified or FDA-cleared commercial procedures are used, laboratories must verify data on the assay performance and assay specific target ranges given by the manufacturers, and their conformity to both analytical and clinical requirements. In addition, conformity to certification or clearance requirements (eg, the completeness of the package insert—does the intended use match with the population to be monitored?) should be reviewed. A summary of the performance characteristics of presently available assays as reported by the manufacturers is shown in Table 2. As mentioned above assay parameters reported by the manufacturer might be different from those observed by the users during routine application.

Validation/verification of methods for ISDs should be performed on samples based on the same matrix and the same

anticoagulant that are intended to be used in routine services. Whole-blood samples are recommended for the TDM of cyclosporine, tacrolimus, sirolimus, and everolimus, whereas plasma is the material of choice for mycophenolic acid (Table 1). Ethylene-diaminetetraacetic acid (EDTA) is the preferred anticoagulant because it minimizes problems with clotting and its use allows for the quantification of multiple immunosuppressive drugs in the sample. Method validation/verification should include experiments with actual patient samples because they reflect the relevant proportions of free and bound drug and of parent drug and drug metabolites. In addition, testing for the effects of comedication and disease conditions should be performed. Spiked samples could be used to supplement the experiments, but one should be aware that they may not provide an accurate assessment of the performance characteristics and demonstrate the robustness of the method if used as the only matrix. Furthermore, it is recommended to include fresh patient materials to the sample set because frozen samples do not allow for the full testing of the sample pretreatment procedure. For instance, sample pretreatment for analysis of cyclosporine, tacrolimus, sirolimus, and everolimus should ensure complete hemolysis of fresh whole-blood samples, but this has already occurred when the whole-blood samples have been frozen. As usual, it is important to make sure that instrument/system suitability testing is conducted regularly during method validation and verification.

Method Crossvalidation

It has to be noted that assay validation can potentially become a self-confirming system and that even after meeting all acceptance criteria, results might be incorrect. This can particularly occur if in-house calibrators have been used that have incorrectly been serially diluted. Hence, measurement of external controls and crossvalidation with peer laboratories is critical before the measurement of clinical TDM samples can commence. Whenever laboratories interchange different validated and/or verified methods performed on qualified platforms, for example, when moving a measurement service between different models of an instrument (eg, for back-up purposes) an assay crossvalidation assay is required. Method crossvalidation should include the analysis of both QC and patient samples. Patient samples should mirror the monitored population.

Validation Reports

All results generated during the method validation/verification should be summarized in a validation report which includes at least information about the experiments performed, the materials and reagents used including calibrators and QC samples, the source of the specimens used, the statistical analysis performed, the analytical performance and the acceptance criteria used for approval. Unexpected results obtained during validation/verification and deviations from the recommended performance must be discussed with regard to their significance to the clinical dose adjustment process.

METHOD PERFORMANCE CHARACTERISTICS TO BE INCLUDED IN THE VALIDATION AND ACCEPTANCE CRITERIA

As mentioned above, validation procedures for methods designed to provide ISD TDM services should follow the detailed procedures described in the guidelines of international scientific societies and governmental agencies whenever feasible. Hence, the following paragraphs will focus on specific issues related to ISD analysis, rather than recapitulating these well-known guidelines. Earlier, IATDMCT recommendations regarding acceptance criteria for analytical method performance have been updated to reflect current clinical needs and technical advances.[8–12,14,39]

Method Specificity for the Parent Drug

The target ranges currently specified by TDM of ISDs are for the parent drug. This is either because of a lack of significant pharmacological activity of drug metabolites or because of a negligible concentration of pharmacologically active metabolites in the specimen compared to the parent drug[5,7–11,13,14] (Table 1). Therefore, analytical methods should be specific for the parent drug determination. If metabolites are present, assay crossreactivity to these analytes should be reported to the user with a statement of clinical relevance. Crossreactivity with drug metabolites determined by a ligand-binding assay leads to overestimation of the drug concentration and is likely to cause too low drug exposure if not taken into account at dose adjustment. No current evidence exists to support the monitoring of single metabolite concentrations of any ISD as a part of a routine TDM service.

In general, even if metabolites do have an ascribed pharmacological activity, analytical methods must be substance specific. If the presence of a particular metabolite should be covered in a specific situation (eg, the acyl glucuronide of mycophenolic acid) its concentration must be evaluated as a separate analyte. That implies that in chromatographic assays, even if mass spectrometry is used as a detector, baseline separation of the chromatographic peaks and individual calibration curves for both analytes are necessary to avoid in-source fragmentation-related bias, particularly if time pressure leads to rapid chromatographic approaches.[100] For ligand-binding assays, the situation is more complicated. Even if 100% crossreactivity of the capture protein to the parent drug is claimed and its bioactive metabolite has been tested in a quantitative manner by the manufacturer during assay validation (which is not the case for any currently available immunoassay for an immunosuppressive drug, Table 2), such results are likely to not be transferable to routine samples, as recently shown for the endogenous analyte 25-OH-vitamin D.[101,102] Hence, results from crossreacting ligand-binding assay must not be seen as the sum of the individual levels of drug and metabolite. On the one hand, even if a metabolite is reported to be pharmacologically active, the in vivo PD and toxicodynamic properties are not necessarily identical with those of the parent drug. Conversely, crossreactivity in an immunoassay is mostly concentration dependent, is not parallel to the parent drug–response curve, and will be affected by factors,

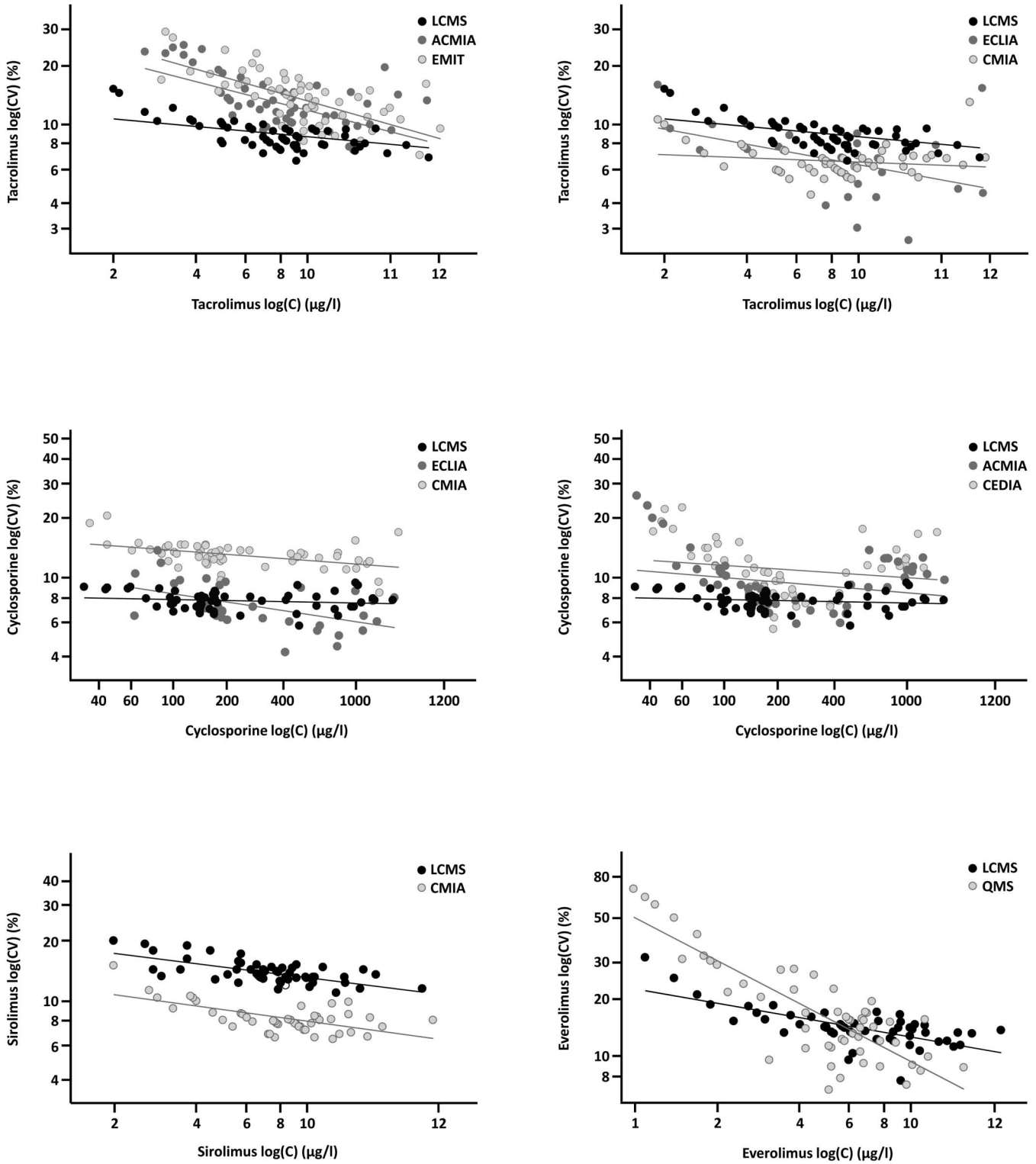

FIGURE 1. Precision analysis of ISD-TDM platforms. Data from all patient and spike samples distributed within the past 60 challenges of the UKNEQAS PT scheme (www.bioanalysis.co.uk) were analyzed. Group means and coefficient of variation (CV) were taken from the PT scheme provider; blank samples were excluded. Average number of participants in the groups were as follows: cyclosporine: LCMS 150, CMIA 110, ECLIA 20, ACMIA 60, EMIT 25, CEDIA 40; tacrolimus: LCMS 180, CLIA 155, ECLIA 20, ACMIA 30, EMIT 20; sirolimus: LCMS 160, CMIA 60; everolimus: LCMS 130, QMS 40.

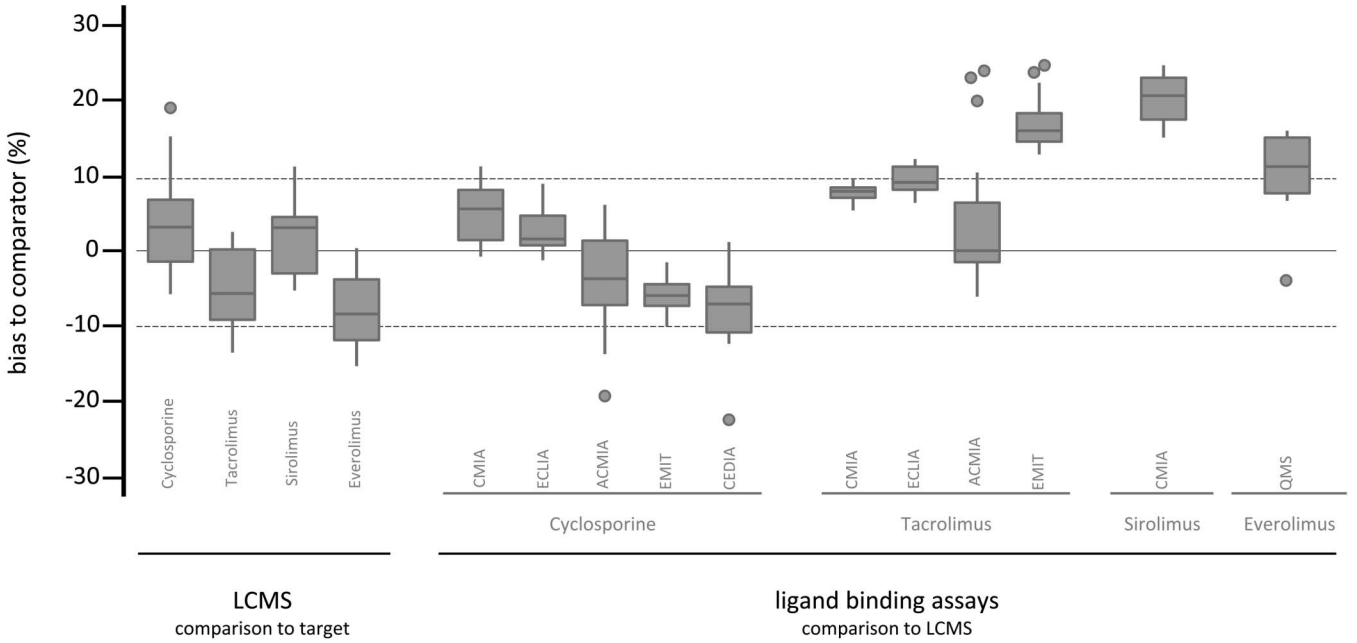

FIGURE 2. Bias analysis of ISD-TDM platforms. Data from patient samples distributed within the past 60 challenges of the UKNEQAS PT scheme (www.bioanalysis.co.uk) were analyzed for the ligand-binding assays, matching spiked samples were analyzed for the LCMS group. Ligand-binding assay group means were compared to the LCMS group mean, LCMS group mean results were compared against the target value issued by the PT scheme provider. Mean analyte concentrations for the LCMS group were as follows (patient samples/spiked samples in mcg/L, number of samples): cyclosporine: 160/206/21; tacrolimus 7.7/7.5/18; sirolimus 7.7/7.8/17; everolimus 5.7/6.3/11. Average number of participants in the groups: cyclosporine: LCMS 150, CMIA 110, ECLIA 20, ACMIA 60, EMIT 25, CEDIA 40; tacrolimus: LCMS 180, CLIA 155, ECLIA 20, ACMIA 30, EMIT 20; sirolimus: LCMS 160, CMIA 60; everolimus: LCMS 130, QMS 40.

such as the metabolization rates, drug–drug interactions, time after dosing and/or clinical disease that affects drug elimination.[103] However, knowledge of the expected relative concentrations of a crossreacting metabolite to the concentration of the drug at the sampling times used in the TDM (Table 1) is important during method development to estimate the potential impact on patient results. To evaluate crossreactivity with the parent drug, either spiked samples with highly purified metabolites at the highest clinically relevant concentrations can be used or the metabolites present in the patient specimen can be measured chromatographically, followed by an estimation of their effects on the immunoassay under evaluation.

Assay-Measurement Range

When developing an analytical method, a working range (the range between LLOQ and ULOQ) that covers the full concentration range expected for each single ISD in patient samples should be aimed for. This concentration range depends on the therapeutic scheme (eg, applied dose or cotherapy) and the TDM strategy (predose concentrations, Cmax, C2, abbreviated or full AUCs). Laboratories should characterize the working range of their method, and if dilution is required to allow high concentrations to be measured, appropriate protocols should be developed and validated. Patient samples with high drug concentrations or spiked whole blood can be diluted. Because the crossreactivity of drug metabolites in immunoassays may be concentration dependent, including actual patient samples for the evaluation of dilution integrity is strongly encouraged if ligand-binding assays are to be used. To be acceptable, dilution integrity should demonstrate accuracy and precision within set criteria (see below). A standard procedure for the reporting of concentrations outside the working range should be available. It must not be overlooked, that in general some immunoassay designs may lead to a "high-dose hook effect" in which the apparent analyte concentration drops to almost zero at very high concentrations.[104] A manufacturer must report to the user about affected products in the product information.

The LLOQ issue is particularly important because very low target ranges for some immunosuppressive drugs have been introduced in an attempt to reduce long-term toxicity.[105] Hence the quantification limit must be geared toward the lower limit of the target range that can be understood as a decision limit and not vice versa. To ensure a reliable determination of such low concentrations and to detect inappropriate low dosing or patient nonadherence, analyte quantification should be at least one-third to half of the lower limit of the target concentration window, for example, an LLOQ close to 1 mcg/L should be achieved for tacrolimus, sirolimus and everolimus assays to allow meaningful TDM at 2–3 mcg/L drug concentrations. Similarly, LLOQs of 20 mcg/L for cyclosporine and 0.2 mg/L for mycophenolic acid procedures should be targeted. Method

imprecision and an inaccuracy of ≤20% at the LLOQ must be demonstrated during validation or revalidation, as stated in several guidelines.

Measurement of free or intracellular drug concentrations is currently not established or recommended for TDM services for any immunosuppressive drug. However, some reports demonstrated promising results with such strategies,[106–108] and the high sensitivity of modern LC-MS/MS instruments enables such analyses. Clearly, a lower LLOQ than those given above would be necessary to quantify free or intracellular ISD concentrations.

Assay Precision

Reproducibility of the results is important to facilitate consistent dosing decisions. Both within-run and between-run precision should be characterized using concentrations corresponding to the within, above, and below the recommended target range defined for the respective single drug. In general, for ISD methods, a CV of ≤10% or even ≤6% (see total analytical error estimate calculation further below in this section) should be aimed for. This requirement refers to between-day imprecision and is based on estimated variations that may result in poor therapeutic decisions.[109,110] It has to be noted that these values are more restrictive than CV ≤15% presented in the EMEA and FDA guidelines,[22,23] which are however not targeting clinical routine measurements.

Assay Accuracy

Measurement accuracy, the closeness of agreement between a single result and the true concentrations of the analyte,[21] has recently received particular attention because a large number of different assays have entered the marketplace. Although they are reproducible, many of these assays generate distinctly different results due to crossreactivity or improper calibration, which is confusing in a clinical setting. Hence, availability of methods that produce unbiased concentration measurements is an important issue. From a clinical viewpoint, efforts should be made that patient samples can be tested with any validated/certified method in any laboratory without any impact on the dosing advice.

Accuracy can be evaluated by means of an analysis of materials with an assigned drug concentration (eg, third-party prepared calibrators, or controls traceable to a well-characterized reference material) and through a comparison with a validated reference method. In addition, the results generated with the method during validation can be compared with those from external proficiency testing. As mentioned above, using authentic patient samples to assess accuracy is essential to uncover potential specimen-related bias (eg, due to matrix effects or crossreactivity to metabolites). Although external "pooled patient" proficiency-testing samples are very useful, they can also underestimate errors that may occur in individual patients because pooling blood tends, on average, to decrease the magnitude of error that might occur for individual patients due to matrix effects. In addition, proficiency-testing samples are typically prepared using leftover material from previous TDM analysis that has sometimes undergone multiple freeze–thaw cycles and/or has been stored under poorly controlled conditions, compromising sample integrity. Therefore, evaluation of the performance of new assay measurements using a chromatographic reference method and actual (nonpooled) samples is advised when first working with patient samples, and when large individual deviations are suspected.[39]

Before an exact-matching isotope-dilution mass spectrometry method reference service is available for ISDs, fully validated LC-MS/MS–based procedures with well-documented assay performance that are specific for the parent drug should be considered as the reference.[111,112] However, both data from external proficiency testing and data from the literature demonstrate distinct differences in the performance of individual LC-MS/MS assays.[111] In addition to a lack of standard reference methods, comparison of analytical procedures is hampered by the absence of certified reference materials. Tacrolimus is the only ISD for which a whole-blood certified reference material is available (ERM-DA110a), and two studies have already shown that this reference material can be used to test the accuracy of methods, and that standardizing the individual laboratory-developed LC-MS/MS procedures can help in minimizing between-method bias originating from a lack of interassay accuracy.[111,112]

Methods should be compared by an unbiased procedure as Deming regression or Passing Bablok regression.[113,114] A comparison should include samples from a wide variety of pathologic conditions that are characteristic for the intended patient population (different transplant types, time post-transplantation, time of blood draw with respect to drug administration, ethnic backgrounds, age groups, etc.) and present a wide range of concentrations including those within, above, and below the recommended target range. In some cases, a separate analysis of specific subsets of the data may be indicated. For example, when verifying the performance of an immunoassay, a separate analysis of data for each organ transplant group gives a better estimate of the effect of drug-to-metabolite ratios that may be due to differences in the formation or elimination of crossreacting metabolites.

Meeting the following criteria compared to a reference procedure is recommended for the acceptability of a method for the selective determination of ISDs:[8,12,14]

1. A linear regression slope within ±10% of the theoretical value of 1.0.
2. A linear regression intercept not significantly different from zero.
3. A standard error for the estimate, Syx ≤10% of the average of the target concentrations.

The number of samples tested must be sufficient to allow for meaningful statistical analysis (n = 40–100), and comparison measurements must comprise several calibration events (n ≥ 3), allowing for an assessment of the intermediate accuracy in the data evaluation. In addition, a data evaluation for the comparison of measurements must include an absolute and relative Bland–Altman plot and an evaluation of the individual measurement bias to ensure that outliers are treated appropriately.[115] Such treatment might include repeated measurement on both platforms to exclude measurement error; investigation of other clinical parameters, including

hemolysis, icterus, and lipemia; plasma exchange experiments to screen for interfering molecular entities; and medical record investigations to evaluate comedications that might, for example, interfere with the metabolism of the monitored drugs and clinical condition (eg, impaired renal function) of the patient.

Precision and trueness (bias) of ISD-TDM methods must be assessed by comparison with meaningful clinical and physiological target ranges.[116] The drug concentration varies with the state of the patient, so target ranges immediately after transplantation may differ from those for long-term transplant patients. Because data on longitudinal intraindividual biological variation of ISDs are still scarce,[117,118] it should be feasible to estimate the analytical variability and bias from clinical TDM goals, for example, to determine a trough concentration range for a patient. If, for example, a tacrolimus target range is set from 8–10 mcg/L, it can be assumed that this range mirrors the intraindividual biological variability for a patient to be clinically meaningful. Because individual measurement points in this range are usually following a Gaussian distribution, this range can be interpreted as a 95% CI ("2S range") around a mean drug concentration of 9 mcg/L. Consequently, the CV associated with this intraindividual biological variability can be assumed to be $CV_I = 5.6\%$. Applying the widely accepted model by Fraser et al ($CV_A < 0.5 \times CV_I$)[119] that derives the desirable analytical imprecision from the intraindividual biological variability,[120] a $CV_A = 2.8\%$ is calculated. Such a CV_A is barely achievable in routine use. Even with a broader target range, for example, 6–12 mcg/L for tacrolimus ($CV_I = 11.2\%$) or 85–135 mcg/L ($CV_I = 11.2\%$) for cyclosporine, the derived $CV_A = 5.6\%$ is a challenging analytical goal. Applying the more TDM-related CV_A model by Glick[121] ($CV_A < 0.1*(C_U - C_L)/C_U$, where U and L are the upper and lower range limits) that focuses on the therapeutic target range as such and is based on the assumption that the analytical error must be significantly smaller than this range, the CV_A to be aimed for is of a similar size ($CV_A = 2.0\%$ for the range 8–10 mcg/L; $CV_A = 5.0\%$ for the range 6–12 mcg/L). If the analytical performance goal estimation for TDM by Fraser[18,122] is applied, which is based on fundamental PK theory, the ISD-TDM CV_A values must also be much below 10%, depending, of course, on the half-life and the dosing regimen for the individual drug. In general, the discussed error estimate for tacrolimus agrees with data for other TDM analytes with similar target ranges.[123,124] Translating the analytical imprecision goal $CV_A = 5.6\%$ into a total analytical error estimate ($TE_A = 1.65* CV_A + B_A$), the allowable analytical bias B_A must be lower than 5.8% if a TE_A goal of 15% is assumed to be feasible. Again, this is a challenging goal particularly if interassay bias values for the ligand-binding assay from metabolite crossreactivity or for laboratory-developed mass-spectrometry installations from calibration heterogeneity are considered (Figs. 1 and 2). In summary, for ISD TDM, a CV_A of approximately 6% should be aimed for. Whenever B_A exceeds the lower percentage range (to be expected if calibration systems depending on different reference standards are used), TE_A will increase such that it is extremely difficult to monitor ISDs even if a clinically rather large target range (eg, 6–12 mcg/L for tacrolimus) would be accepted.

Specific Technique-Related Pitfalls and Interference

Method validation should include an evaluation of specific technique-related pitfalls, which might cause errors in drug concentration estimates. To test for such effects, sample drug concentrations should be adjusted to near the medical decision limit. Examples for immunoassays are interferences due to crossreactivity with other drugs and metabolites, reactions with heterophilic antibodies, antibodies directed against the binding antibody, and the effects of endogenous factors, such as hematocrit, albumin, bilirubin, and triglycerides.[125,126] For instance, the results generated by the tacrolimus IMX II-MEIA, which has now been removed from the market, were shown to be strongly affected by the sample hematocrit.[117,127,128] A number of false-positive results have been reported for immunoassays based on the ACMIA test format, most probably due to the problems with heterophilic antibodies.[129–131] For LC-MS/MS procedures, a major pitfall is the possible presence of matrix effect (eg, ion suppression or ion induction) that is particularly risky when using electrospray ionization (ESI).[132–134] Both the effects of hydrophilic and lipophilic matrix components as salts or phospholipids, respectively, are an issue.[135,136] High extraction and chromatographic separation efficacy (eg, using SPE or 2D-chromatography) and the use of stable isotope internal standards can minimize matrix effects.[135] Other possible problems that can compromise results and that must be addressed during the validation of LC-MS/MS procedures include in-source fragmentation (demonstrated, eg, by the glucuronide metabolite of mycophenolic acid glucuronide [MPAG][81]), interference with internal standard transitions by drug metabolites (eg, when using cyclosporine D as an internal standard for cyclosporine analysis[61]) or interference with drug analysis because of contamination of the internal standard (eg, the analysis of sirolimus in a multiplex assay using $^{13}C_2D_4$-everolimus as the internal standard[137]). In addition, factors such as isotopic purity issues, cross-talk between MS/MS channels, and isotopic integrity should be considered while validating a LC-MS/MS method.

Both analysts and clinicians should be aware of possible pitfalls of the methods used when interpreting patient results.[135] The laboratories providing services for TDM of ISDs are responsible to continuously inform/educate their clinical partners about issues that might affect the performance of a particular method.

Carryover

Carryover can occur during analysis and compromise accurate measurements. Therefore, it should be evaluated as part of the validation of measurement procedures for ISDs. If carryover is detected in a blank sample processed immediately after a sample with a drug concentration close to the highest expected clinical concentration, it should not be greater than 20% of the LLOQ.

Stability Issues

As previously mentioned, method validation should include an assessment of the short-term and long-term sample

stability and the stability of stock solutions, sample extracts, and reagents. Stability experiments should mimic the local conditions under which samples are collected, transported, stored, and processed as closely as possible. Expiration dates should be clearly marked and analysts should strictly adhere to those. Laboratories are advised to setup specific procedures for temperature control during the transport, storage, and handling of samples (Table 1).

Effects of Changing Solvent and Reagent Suppliers or Lots, Tubes, or Vials

Changing solvent and reagent suppliers or lots, tubes, or vials may compromise analytical reliability; therefore their quality should be always demonstrated before use for patient sample analysis.

Dried Blood Spot Sampling Specific Issues

DBS represents a new intensively investigated strategy for the TDM of ISDs to facilitate outpatient management.[138] However, this sampling strategy has some specific challenges[139,140] that should be considered during method validation. The major drawback is that the volume of the blood sample cannot exactly be measured. Thus, calculation of ISD concentration is based on the assumption that the punched out filter paper blood spot is saturated and always contains the same blood volume. This assumption may not always be correct. Important factors that may affect the accuracy of the results include sampling practice, the size and drying time of the spot, the temperature and humidity of the environment, the type of Dried Blood Spot (DBS) paper and the on-card storage stability. Hematocrit[141] plays a particularly significant role for DBS because different hematocrit values are associated with different viscosities, impacting on the spread of the blood on the DBS paper. As shown by Koster et al,[142] the specific combination of DBS samples with extremely high drug concentrations and extremely low hematocrit values is challenging. For specifics related to validation of methods for DBS, the reader can refer to the recommendations of the European Bioanalysis Forum.[143]

METHOD LIFE-CYCLE MANAGEMENT

The long-term consistency of the results generated with a method is of high importance in transplantation medicine. ISDs are used in life-long treatment of most transplant patients; individualized target ranges are usually established post-transplantation with a certain method (eg, LC-MS/MS at the transplantation center). Usually patients stay attached with their transplantation center for several years with visiting time intervals from some weeks to several months. Hence, the ISD-TDM platform must be stable over such times; any long-term inconsistency of results may negatively impact dosing decisions and the patient outcome. Therefore, a method life-cycle management should be established to guarantee that analytical performance documented during method validation is continuously reproduced.

Stable analytical performance over time is based on a robust assay performed by well-trained personnel on well-maintained instruments. Assay bias and assay imprecision are kept low by monitoring the assay while it is being performed and by following good laboratory practices. Measures to avoid calibration bias include participation in an external quality assurance program, the use of external commercial calibrators, QC materials (preferably from different manufacturers), and the availability of certified reference materials and reference methods for all ISDs.

A rigorous internal quality assurance program that includes both system suitability testing (control of temperature in different compartments of the instrument, signal accuracy, signal stability signal intensity, signal recording, retention times, etc.), and revalidation of critical analytical parameters for existing methods is strongly recommended. Laboratories should have established protocols for these procedures that should be conducted on a regular basis to ensure continuous fitness with regard to analytical specifications and clinical requirements. For instance, the introduction of new therapies to the intended patient population or extension of this population to new disease groups may introduce challenges that were not present during the initial method validation. For example, experience from the Symphony clinical trial[144] demonstrated that although the intended tacrolimus concentrations were in the range 3–7 mcg/L, most patients had actually been at or above the top concentration in this range. This was partly because the analytical method used by most centers to measure the drug (MEIA) was never designed to measure tacrolimus at such low concentrations.[145]

Guidance on how to design and implement an appropriate quality assurance program is usually provided by national laboratory regulations, such as the Clinical Laboratory Improvement Amendments (CLIA) in the US or the RiliBÄK (Quality Standards for Medical Laboratories of the German Chamber of Physicians) in Germany.[146] Furthermore, ISO15189 in its current version (ISO15189:2012, version 2014-08-15) specifies requirements for the quality and competence in a medical laboratory. The QC strategy of every laboratory should be to determine when a method is out of control, the reasons why, and when it returns under control. For example, a plan including types and levels of QC materials to be run, frequency of performing QC runs, QC acceptance criteria, and statistical evaluation of QC results should be established to reliably detect both systematic (trends or shifts) and random errors. Appropriate control levels should concentrations within, above, and below the recommended target ranges with the lowest concentration not exceeding 3 times the LLOQ. Audits and reviews aiming for improvement should be conducted on a regular basis to allow for timely detection of deviations from the specifications. All procedures and measures to monitor, evaluate, and guarantee stable performance of the analytical measuring system, as well as interventions to resolve "out-of-control" situations should be carefully documented. Profound documentation serves a dual purpose: it documents the performance of the assay (particularly long-term) and will facilitate troubleshooting.[147,148]

In addition to the internal quality assurance program, laboratories performing ISD TDM must participate in an external quality assurance program to allow continuous crossvalidation and proof of analytical quality (eg, Analytical

Services International Ltd,[91] CAP[149]). In addition to spiked samples, proficiency-testing samples should include samples that do not contain the drugs of interest and particularly pooled transplant patient samples from patients after the transplantation of different organs, as well as from nontransplanted patients, if applicable.

Continuous education and training of TDM laboratory personnel is an integral part of ensuring a high level of analytical quality. Therefore, establishing programs to maintain an adequate educational and training level of the personnel involved in analysis and reporting or interpreting the results is strongly recommended. Finally, it should be reminded that the clinical effectiveness of TDM (of ISDs particularly) largely depends also on the respect of the sampling hours and of any preanalytical recommendations as correct sampling from catheter sytsems.[150,151] Continuous education should therefore ideally include the nursing staff and health care professionals.

CONCLUSIONS

Reliable performance of analytical methods that are used for TDM services focusing on immunosuppressive therapy in transplantation is essential to enable proper therapeutic decisions and dose adjustment. This document was developed on behalf of the Immunosuppressive Drugs Scientific Committee of IATDMCT. It aims to provide recommendations for the establishment and maintenance of appropriate laboratory practices, to adequately reflect current clinical needs for advanced optimization and individualization of the therapy with ISDs to allow for prolonged graft survival and an improved quality of life of organ recipients. These recommendations address all phases of the analytical procedure life cycle, including method design, method validation and performance verification; the definition of appropriate acceptance criteria for analytical performance; risk assessment; and method life cycle management. Regarding method validation, a proposal on how to adapt the recognized guidelines published by international scientific societies and governmental agencies, for the analysis of ISDs, has been provided. Both specifics related to LDT and commercial tests at the analytical site are covered. Actions aimed at improving the consistency of the reported results (including between-method, between-laboratory, and over time consistency) have the highest priority. These include but are not limited to the advanced standardization of methods and testing practices, the development of appropriate reference materials and reference methods, the improved availability of actual patient sample-based materials from external proficiency testing, the enhanced level of compliance to internationally accepted laboratory practice guidelines and to the defined TDM-specific recommendations through rigorous education and continual training. We are convinced that at the current state especially LC-MS/MS–based LDT systems clearly need methodological consolidation and guidance. Possible new areas of development, such as the measurement of intracellular concentrations, new sampling strategies, method multiplexing, or point of care testing, may need to be further elaborated in the current recommendations in the future to cover specific requirements if clinical implementation is intended.

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