

Immunosuppressant drug monitoring – a routine undertaking?¹⁾

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Abstract

The quantitative assessment of immunosuppressant drug levels is still one of the most challenging therapeutic drug monitoring procedures in clinical routine. During the past years, several technical developments matured to usable methods. In addition to immunoassays, liquid chromatography-tandem mass spectrometry has become a key method in immunosuppressant therapeutic drug monitoring. This overview should aid in understanding the advantages and disadvantages of the various assays and methods. UK-NEQAS proficiency testing results are used for an inter-assay comparison approach.

Keywords: immunoassays; immunosuppressant drugs; liquid chromatography-tandem mass spectrometry (LC-MS/MS); proficiency testing; tandem mass spectrometry; therapeutic drug monitoring.

Introduction

It is indisputable and therefore requires no further explanation, that therapeutic drug monitoring (TDM) of immunosuppressant medications plays a central role in the diagnostic care of transplant recipients. From the first post-operative days to outpatient aftercare of long-term stable transplanted patients immunosuppressant TDM has had a decisive part in the rising survival rates of organs and recipients observed over the last years [1–5], although the difficulties of chronic allograft failure (CAF) and the occurrence of massive long-term side effects continue to represent an as yet unsolved problem [6–8].

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What is less clear from the perspective of the laboratory and what is easily forgotten in the light of the accomplishments of the last decade is the ever recurring question, when confronted with new technologies, which means to choose for the creation of a monitoring program ideally adjusted to clinical needs. Although the assay selection as a rule will primarily depend on local conditions and the equipment of the laboratory, it must not be forgotten, that in regard to data quality the selected procedure must be up to the demands of the task.

Comparable to the detection of many other drugs, the analyst has mainly immunologic or chromatographic procedures at his disposal to monitor immunosuppressants. The realization of immunologic procedures is the domain of large industrial manufacturers and has led to a series of more or less automated IVD-CE [9] certified assay applications on high throughput machines. In the case of chromatographic analysis salvation is usually sought in in-house development because of a lack of alternatives. Especially in the last few years LC-MS/MS – the use of tandem mass spectrometry (MS/MS) as the detector in liquid chromatography (LC) – has experienced a brilliant upswing [10–13]; a development that reflects the wide-spread use of this technology in the pharmaceutical industry. For example, in the area of immunosuppressants the clinical testing and market entry of everolimus a few years ago (approved in 2004) was accompanied by LC-MS/MS-based drug level measurement (including the definition of therapeutic ranges), while not long before immunoassays have been developed for TDM along with the market – introduction of tacrolimus and sirolimus (approved 1996 and 2001).

This survey will attempt to show the general advantages and disadvantages of the different analysis platforms. Besides methodic outlines we examine the fundamental problems of measurement platforms and provide substantiation with examples from practice and literature. From a technological perspective therapeutic drug monitoring of immunosuppressants cannot and should not be looked at in isolation. Therefore, we also deal with other examples from TDM and from the quantitative analysis of endocrine metabolites.

Requirements

A low therapeutic quotient and the resulting narrow therapeutic bandwidths (factor approx. 2–5), drastic side effects when quitting these (danger of rejection or toxic side effects), massive drug interactions as well as high intra- and inter-individual variability in metabolization resulting in the loss

of the dosage-exposure relationship require tight monitoring to control whole blood-drug levels in the therapy with cyclosporine A, tacrolimus, sirolimus, everolimus and the serum-drug levels in the therapy with mycophenolic acid. Particularly during the first few weeks following transplantation finding the right dosage is often difficult. Accordingly, clinicians demand reports to be delivered promptly; typically total turnaround times of no more than 3 to 4 h are desired. Measurements must be precise and true; only the smallest possible laboratory error – a negligible imprecision and a minimal inaccuracy – permit dosage adjustments at a narrow therapeutic target range. For example, at an everolimus level of $C_0=5$ ng/mL (recommended therapeutic range 3–8 ng/mL) [14, 15] and a relative measuring error of $CV=10\%$ (inter-assay CV) the relevant critical difference for the clinical decision finding ($D_{crit} \sim 2.8 \cdot SD$ for $p < 0.05$) [16] is $D_{crit} \sim 1.4$ ng/mL which – even without allowing for biological variability – already covers approx. 55% of the therapeutic band.

When systematic (e.g., calibration bias) and intra-individual (biologic) variability are added to this measurement dispersion entirely common in the practice, analysis platforms of this kind are no longer able to meet clinical requirements [17, 18]. Less precise (high variability of results) and poorly calibrated (systematic method bias) measurement systems leave no room for therapeutic interventions, such as dosage adjustments, within the bounds of the therapeutic range.

The fact that modern therapeutic schemes (e.g., combination therapies, dosage reduction in cases of partial immunologic tolerance) [19, 20] demand to measure lower drug levels more correctly than in the past, represents an additional challenge to be met by all commercial providers and manufacturers of “in-house assays” alike. Generally it can

be said that therapy-appropriate immunosuppressant levels (LC-MS/MS values) < 3 ng/mL for tacrolimus, sirolimus and everolimus or < 75 ng/mL for cyclosporine A are no longer a rarity. For example, to deal with this aspect a recent consensus document for tacrolimus TDM demands that test systems must show a quantification limit (functional sensitivity) of at least 1 ng/mL [21]. Presumably, older consensus documents will undergo analogous adaptations in the near future [22, 23].

New immunoassays – new hope?

The use of immunoassays for the quantitative detection of small organic molecules (medications, drugs, endogenous analytes) presents a series of analytical problems that cannot always be solved satisfactorily. In many cases – quotable examples reach far beyond immunosuppressants – poorly selected or insufficiently developed antibodies cause the additional detection of structurally similar analytes. This includes metabolites of the target substance (e.g., hydroxylated cyclosporine A metabolite [17], mycophenolic acid-glucuronide [24, 25], 17-OHP-sulphate in 17-OHP assays [26]) as well as structural analogs (e.g., cortisone in cortisol assays [27, 28], oleandrin and other steroids in digoxin assays [29, 30]).

When a certain structural dispersion in antigen recognition is not expressly desired (as is the case, for example, with toxicological group tests), this cross reactivity provides unclear analysis results (Figure 1). In comparison to substance-specific measuring methods like GC-MS, LC-MS or enzymatic methods, it systematically simulates incorrect higher analyte concentrations, since the recorded signal

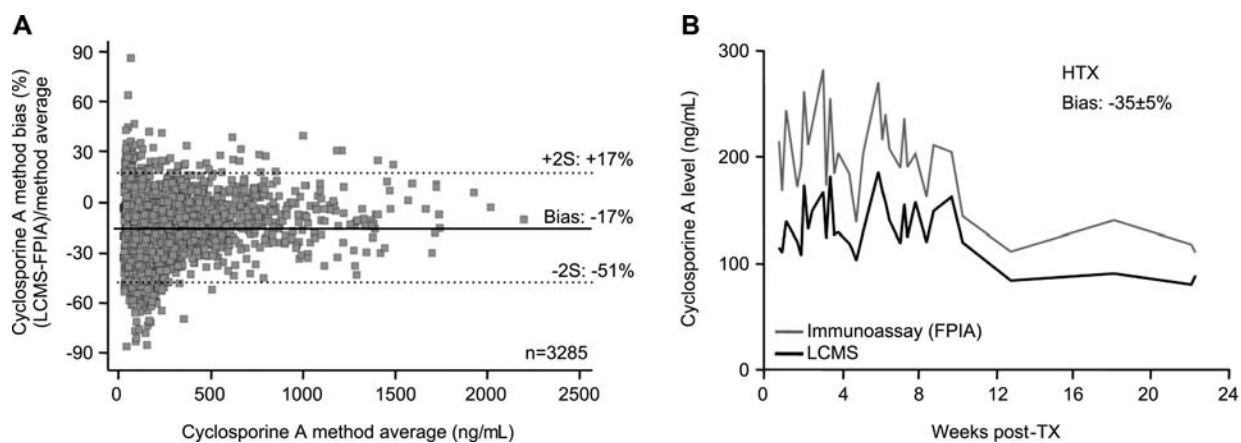


Figure 1 Global bias analysis (A) and individual patient profile (B) as tools for the evaluation of deviations from measured values. (A) The comparison of two analysis systems with the Bland and Altman plot [31] shows that the relative measuring error across the collective (n 3285, C0 and C2 level measurements) is a function of the quantity being measured. Further, an average systematic measurement deviation can be observed (the LC-MS/MS platform on average measures 17% lower than the immunoassay). However, in the clinically relevant measuring ranges (trough level < 400 ng/mL) the collective exhibits a dispersion of such breadth (2S range reaches from -51% to $+17\%$ bias), that a reasonable communication of the “conversion factor” or a “correction formula” is not possible. (B) Individual patient profiles exhibit an extraordinary stable intra-individual bias. With $2S=10\%$ the fluctuation range of the drug levels, which in this case include the complete post-TX period including outpatient aftercare, is far less than the global fluctuation range of $2S=34\%$. The communication of such data will more likely provide sufficient safety in case of an assay change.

stems from the detection of the target analyte and an unclear numbers of metabolites or analogs. Due to substance-specific cross reactivities and the method bias [31] resulting thereof the interpretation of the analysis result can be correspondingly poor; this can lead to clinical consequences in the aftermath [32, 33].

A further grave disadvantage of immunoassays is that the non-covalent antibody-antigen interaction must take place under more or less physiologic conditions. However, since analytes (because of their lipophilia) are mostly bound to proteins, such as albumin or the steroid hormone binding protein (SHBP), or are imbedded in cellular components (e.g., immunosuppressants in erythrocyte membranes), they must be released from these bonds ahead of detection through the antibody. Since a release through destruction of the binding protein or the cell is always non-physiological, the release must either be attempted through displacement or physiological measurement conditions must be abandoned [34]. This does not play a role in assays based on chromatographic methods, since measurements are performed under non-physiological conditions anyhow. Consequently, a multitude of release strategies can be pursued.

The use of displacement reactions (e.g., with the MPA-MEIA test format [35, 36]) can lead to systematic erroneous measurements whenever there is a change in the binding strength of the analytes to the binding protein (e.g., because of mutations). It has also been shown that this sample preparation strategy makes it impossible to perform measurements if the sample matrix has changed. Hence, standard addition experiments for validating analytical recovery or for comparison with other analysis systems are hardly feasible.

During the past years various immunoassay manufacturers have introduced new assay formats of varying quality (Table 1). The introduction of chemiluminescent magnetic micro-particle immunoassay (CMIA) for tacrolimus, sirolimus and cyclosporine A replaces the fluorescence polarization immunoassay (FPIA) and microparticle enzyme immunoassay (MEIA) test formats by the same manufacturer, that, fraught with diverse analytical problems, no longer fulfill the requirements of the modern TDM of immunosuppressants.

For example, the tacrolimus MEIA assay, implemented on the IMX platform, demonstrated not only insufficient sensitivity and a non-communicated limit of quantification (LOQ) [37], but also an additional unusual analytical problem. It was shown repeatedly [37–41], that the difference of the IMX II-MEIA results to the reference method LC-MS/MS depends on the hematocrit (HCT) of the sample. With pathologically lowered HCT (<35%) MEIA results are systematically higher than LC-MS/MS results (simulating a false high tacrolimus level); with a normal HCT MEIA results are systematically lower than LC-MS/MS results (Figure 2). The overestimation at a low level of HCT is so severe, that measured values of up to 4.5 ng/mL (n=68; median 2.2 ng/mL; 95th percentile 3.4 ng/mL) can be achieved in tacrolimus-free blood samples with a lowered hematocrit (HCT <30%) [37]. The cause for this assay behavior has remained unclear, the consequences, however, are clear – patient management with this kind of TDM assay is extraordinarily difficult; misinterpretations can easily occur.

The new CMIA tacrolimus test ensures that there is no want of accuracy with varying HCT and that the assay sensitivity (LOQ <1 ng/mL) meets the requirements of the new consensus statement [21]. The test performs well in proficiency testing (see below), comparability of the drug levels with LC-MS/MS is very good (Figure 3A). The new sirolimus CMIA also demonstrates acceptable analytical performance data [42], while for the cyclosporine CMIA there are as yet hardly any comparison data to LC-MS/MS platforms. However, first studies allow to conclude a non-negligible bias to LC-MS/MS exists [43–45]. It therefore seems safe to assume that the antibody development for the cyclosporine CMIA has not progressed as far as for the tacrolimus CMIA, for which a new antibody has evidently been developed [46]. With all CMIA assays it must be critically noted that for these test formats the sample preparation from whole blood is extremely labor-intensive (no subjective improvement to the TDX/IMX formats) and can exceed that of a LC-MS/MS assays [47].

In the last few years another also recently introduced tacrolimus assay (antibody conjugated magnetic immunoassay test format, ACMIA), offering easier sample preparation, has been repeatedly reported to give significant false positive results in rare cases [48–50]. If not discovered through careful consideration of both diagnostic and clinical information or through control with another assay such data can have grave consequences (e.g., discontinuation of the medication up to the start of a detectable organ rejection reaction). While the exact mechanism of this defect is as yet unclear, various control experiments have led to the assumption that this is an immunologic problem. It could possibly be a question of heterophile antibodies; it also was shown that high rheumatoid factor titers can lead to high tacrolimus levels [51]. Furthermore, in inter-laboratory testing this assay proved to have massive problems with trueness and accuracy of analysis results (Figure 3A, Table 1).

The partially good performance data of the new immunoassays must not obscure the fact that all these measurement platforms, which are based on an antigen/antibody interaction, exhibit general analytical problems, some of which we mentioned in the previous paragraphs. Besides these analytical limitations the use of an immunoassay often is strictly limited to the use of pure human sample material, which can lead to problems in standard addition experiments and the use of non-human control and reference materials. In many cases, however, the calibration and control material produced by the assay manufacturers themselves and distributed with the kits often consist of non-human surrogate materials. As a consequence the value determination in these matrices often is more of a value assignment, as has been proven by the results of interlaboratory tests (Figures 3 and 4; Table 1). Since antibodies are of biological origin, poor batch consistency of the reagents is a further and not to be underestimated problem. The result could be a sudden loss of assay repeatability and trueness. The possible existence of antibodies that are directed against the binding antibody [e.g., human anti-mouse antibody (HAMA)] represents a further never to be ruled out danger in the use of immunoassays. This prob-

Table 1 Practice-determined trueness and accuracy data (precision) of the most important immunosuppressive test systems. Excerpt from UK-NEQAS inter-laboratory test data (Prof. David Holt) end of 2009/beginning of 2010. Trueness relates to weighed portion.

Analyte	Test name	Manufacturer	Sample 1				Sample 2			
			Concentration, ng/mL	Trueness, % weighed portion	Accuracy, % CV	Participants, n	Concentration, ng/mL	Trueness, % weighed portion	Accuracy, % CV	Participants, n
Cyclosporine	FPIA AxSym™	Abbott	77.9	97.4	11.0	43	204.9	102.5	8.1	43
	FPIA TDX™	Abbott	86.6	108.3	10.2	50	212.4	106.2	6.6	50
	CMIA Architect™	Abbott	85.3	106.6	9.1	66	203.9	102.0	10.3	66
	EMIT®	Siemens	82.4	103.0	13.4	64	203.9	102.0	10.2	64
	ACMIA-Dimension®	Siemens	70.2	87.7	15.7	139	205.6	102.8	6.5	139
	CEDIA® PLUS	Thermo-Fisher	65.4	81.7	17.4	69	195.5	97.8	9.3	69
Tacrolimus	HPLC-MS	–	82.5	103.1	9.3	91	202.7	101.3	6.7	91
	MEIA IMX®	Abbott	3.5	116.6	16.1	76	10.3	103.0	8.7	76
	CMIA Architect™	Abbott	3.2	106.6	9.3	82	10.6	106.0	5.9	82
	EMIT®	Siemens	4.1	136.6	23.9	45	11.3	113.0	13.4	45
	ACMIA-Dimension®	Siemens	3.5	116.6	21.2	78	10.5	105.0	10.6	78
	CEDIA® PLUS	Thermo-Fisher	3.5	116.6	40.5	13	9.7	97.0	14.6	13
Sirolimus	HPLC-MS	–	3.3	110.0	9.1	107	10.6	106.0	7.4	107
	MEIA IMX®	Abbott	1.4	33.4	37.7	46	4.3	53.7	11.1	46
	CMIA Architect™	Abbott	2.6	86.7	7.8	26	6.3	78.7	4.7	26
Everolimus	HPLC-MS	–	2.5	83.4	17.7	107	6.4	80.0	13.5	107
	FPIA	Thermo-Fisher	2.2	110.0	68.4	52	4.3	86.0	36.8	52
MPA	HPLC-MS	–	1.7	85.0	20.0	68	4.3	86.0	14.2	68
	EMIT®	Siemens	4.2	105.0	7.6	53	13.4	111.7	11.8	49
	HPLC-UV	–	3.9	97.5	7.9	47	12.1	100.8	7.9	47
	HPLC-MS	–	4.0	100.0	11.9	37	12.3	102.5	14.7	38

Data base: Cyclosporine A: UK-NEQAS RV 310 (1/2010) 80 or 200 ng/mL; Tacrolimus: UK-NEQAS RV 175 (12/2009) 3 or 10 ng/mL; Sirolimus: UK-NEQAS RV 132 (12/2009) 3 or 8 ng/mL; Everolimus: UK-NEQAS RV 52 (1/2010) 2 or 5 ng/mL; MPA UK-NEQAS RV 51 (12/2009) 4 ng/mL; UK-NEQAS RV 50 (9/2009) 12 ng/mL. All data retrieved from <http://www.bioanalytics.co.uk>, 2/2010.

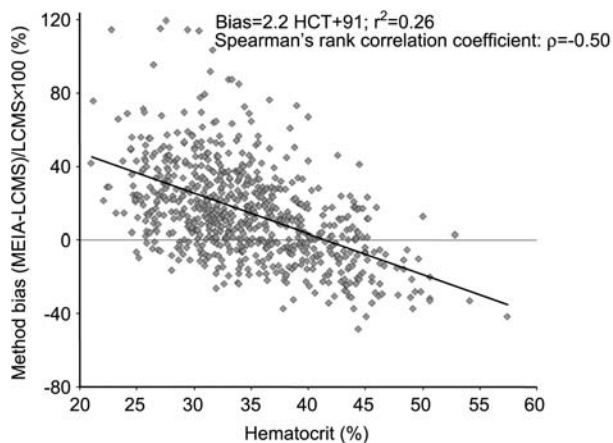


Figure 2 A method comparison between the tacrolimus IMX-II assay (MEIA format) and the LC-MS/MS shows a striking connection between method bias and hematocrit (HCT) of the sample. While the MEIA tacrolimus level is overestimated in samples with a low HCT, levels are underestimated in samples with normal HCT [37].

lem, of course, also is not limited to immunosuppressant TDM assays.

Special case MPA – TDM

Besides the excellent chromatographic methods that are easily realizable, but require manual effort (commercial HPLC-

UV solutions are available), there are two immunoassays (EMIT or CEDIA format) as well as a new kind of enzyme inhibition assay (EIA, Roche) for monitoring mycophenolic acid exposure, which has been advised in recent consensus documents [52, 53], but has also been critically questioned from other quarters [54]. The latter is based on the in vitro inhibition of inosine monophosphate dehydrogenase II (IMPD II), the in vivo MPA effector enzyme of the MPA action. Recombinant IMPD II is used to track the reductive NADH-generating reaction of inosine monophosphate to xanthosine monophosphate. In the presence of MPA the reaction is inhibited, NADH formation is diminished. Both immunoassays have distinct analytical problems; besides limited linearities it has been observed that cross reactivities cause massive, strongly fluctuating overestimations of levels in comparison to chromatographic methods [55, 56]. In contrast, the EIA correlates extraordinarily well with LC methods [57, 58].

LC-MS/MS – a routine method?

The last decade has brought immense technologic developments in the area of LC coupling to tandem mass spectrometry – particularly in the area of ion sources and ion selectors [59, 60]. No end to this development is in sight, even the possibilities in the automation of facilities and the connection to modern laboratory-electronic data processing systems seem inexhaustible [12]. Almost every market leader in the area of instrumental bioanalytics (e.g., AB Sciex, Agilent, Bruker, Thermo-Fisher, Waters) provides a sufficient

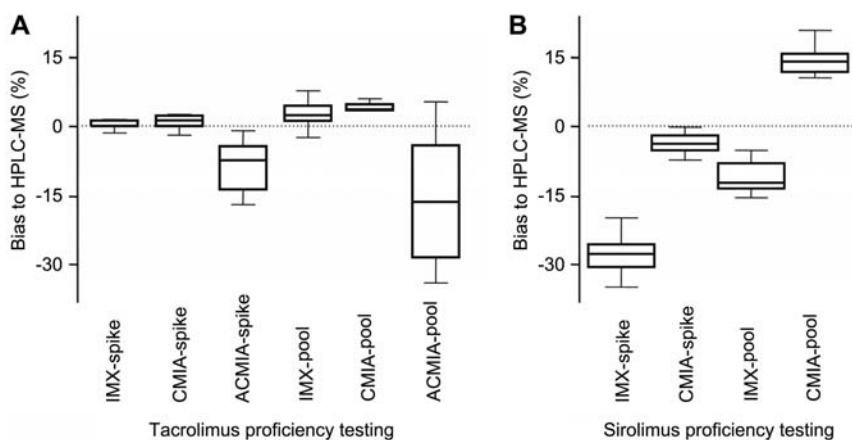


Figure 3 Method bias of some immunoassays to LC-MS/MS. Tacrolimus (A) and sirolimus (B) group results of the UK-NEQAS inter-laboratory tests of 2008/2009.

(A) Bias of the tacrolimus platforms. Spike samples show that the calibration of the MEIA and CMIA platforms was matched to the LC-MS/MS. Accordingly the measurements of pool samples show a (small) method bias, which presumably can be attributed to cross reactions with tacrolimus metabolites. The ACMIA assay cannot recover the target concentrations of the spike samples, this underestimation of levels also manifests itself in the patient pool samples. Data base 10 spike samples (LC-MS/MS value: 8.8 ± 1.3 ng/mL), 15 patient pool samples (LC-MS/MS value: 8.8 ± 0.3 ng/mL). (B) Bias of sirolimus platforms. While with the MEIA assay the method bias in the quantification of patient pool samples is low because of the massive negative calibration bias to LC-MS/MS, the differently calibrated CMIA assay shows a distinct overestimation of the patient pool samples when compared to LC-MS/MS. Since the relative bias difference between spike and pool samples is practically identical (~20%), it may be assumed, that identical or at least very similar primary antibodies are being used. Data base 9 spike samples (LC-MS/MS value: 9.9 ± 2.4 ng/mL), 12 patient pool samples (LC-MS/MS value: 10.5 ± 1.8 ng/mL).

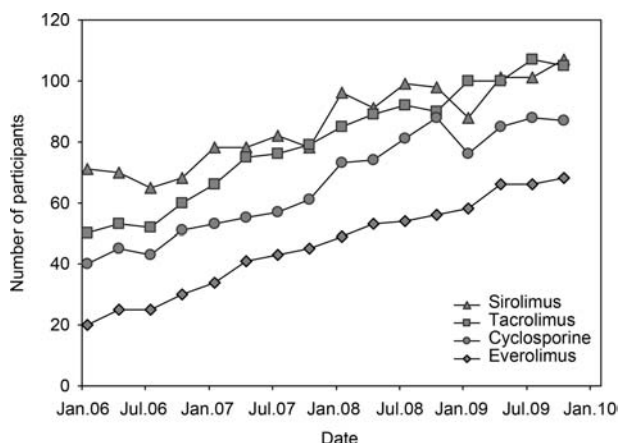


Figure 4 Numerical development of the LC-MS sub-group of the UK-NEQAS PT scheme.

Within the last 4 years a strong increase in LC-MS/MS installations was registered. The approx. 100 LC-MS/MS centers, which at present communicate the results of cyclosporine and tacrolimus inter-laboratory tests, correspond to ~20% and ~27% of the total collective, respectively. At present most of the participants in the inter-laboratory tests use LC-MS/MS for TDM of sirolimus and everolimus (sirolimus ~60%, everolimus ~57%).

MS portfolio to ensure LC-MS/MS analytics in the application target range (quantification limits <1 ng/mL). However, providers show a tendency to present their lower-performing (and therefore cheaper) instruments in a tandem MS palette as sufficiently sensitive for immunosuppressant TDM. Regrettably it has been found again and again that this optimistic view often cannot be confirmed when these instruments are used under routine conditions in the clinic.

During the last years two distinctly different LC-MS/MS instrument configurations have emerged as suitable for immunosuppressant TDM. In each case sample preparation by precipitation of cellular components and serum proteins precedes chromatographic analysis. Similar to some immunoassays, a mixture of an aqueous zinc sulfate solution with methanol has proven itself here as well. The subsequent LC separation of the analytes also serves to purify the sample. In this step the immunosuppressants are separated on the one hand from hydrophilic non-precipitated matrix components (e.g., zinc sulfate) and on the other hand from lipophilic matrix components (e.g., phospholipides). It is urgently advised to ensure a sufficient chromatographic separation of metabolites, since they have been shown to produce interferences [61, 62]. Sufficiently good assay results can be achieved with only one single, simple chromatographic step (LC-MS/MS) as long as important details are taken into account. Under all circumstances trueness, accuracy and sensitivity of the assay should be demonstrated on a sufficient number of patient samples. In our view, however, the use of an additional, also easily realizable chromatographic dimension (online-SPE-LC-MS/MS) [63, 64] without a doubt represents the analytical “state of the art” in immunosuppressant TDM. Nowadays “triple-quadrupole” (synonym

tandem) MS instruments are used almost exclusively for the detection and quantification of analytes. The detection of analytes is performed in the “selected reaction monitoring” (SRM, synonym MRM) mode. Depending on which instrumentation is used, an analysis can be completed within 2–4 min.

At present no MS manufacturer can offer complete solutions that meet the high analytical demands of a routine laboratory. None of the mass spectrometers, let alone the complex LC-MS/MS instrument combination, is IVD-CE certified. Generally, there is no bidirectional connection of the MS control and evaluation software to the laboratory’s electronic data processing. The use of self-generated scripts for the transmission of work lists to the MS or for retransmission of the measured values continues to be common practice. With few exceptions (MassTrak Assay by Waters and MassTox Assay by Chromsystems, both with IVD-CE label) the manufacture of consumables (mobile solvents, precipitants) is in the hands of the local laboratory. Accordingly costly is the establishment and validation of an “in house” LC-MS/MS installation which should adhere to prevailing recommendations and guidelines of international forums, such as CLSI (www.clsi.org), FDA (www.fda.gov) or ICH (www.ich.org). When selecting equipment [design qualification (DQ)] and furnishing the workplace [installation qualification (IQ)] it is necessary to take into account LC-MS/MS-specific requirements, which may not always be easy to implement in the routine laboratory [12]. Only after equipment has been successfully installed [possibly documented by an operation qualification (OQ)] a laboratory can start to set up an assay [performance qualification (PQ)]. Once the limitations of the assay are laid down, a subsequent validation and a risk analysis usually conclude the months-long process of a LC-MS/MS immunosuppressant TDM platform establishment. It must not be forgotten that a frequently observed considerable method bias towards immunologic methods might necessitate a parallel measurement phase of several weeks or even months, to accustom the clients to the new measured values (Figure 1) [64, 65].

The most significant analytic problem of mass spectrometry has become known beyond its immediate circle of users under the catch word ion suppression. It is generally considered to be the greatest disadvantage in the use of mass spectrometry as a detector in bioanalysis [66, 67]. Ion suppression stems from various processes taking place in the ion source (this is the technically complex connector between chromatography and mass spectrometry [60]), which can lead to a complete loss of ionization or to a change in the ionization yield of the analyte molecules (“ion yield attenuation”). Matrix components present in the MS source simultaneously with the analyte are the causative agents in all cases. The elution of detergents, salts or of mobile solvents of incorrect composition (e.g., high proportion of water instead of methanol) usually results in a complete collapse of ionization. These effects can last for dozens of seconds and usually concern the period of elution of hydrophilic sample components (matrix, sample preparation solution). Modulations of the analyte-ion yield can occur at any time, and

require the simultaneous elution of interfering matrix components (metabolite, xenobiotics ...). A signal decrease of the observed analyte can be expected with molecules that represent good ion acceptors, while ion donors can lead to a signal increase. Since fluctuations in the ion yield are concentration-dependent effects, higher concentrated analytes suppress less populated analytes. Particularly with insufficiently selective LC methods or with FIA-MS/MS (flow injection analysis MS/MS) this is a problem that must not be ignored.

The use of internal standards (IS) can counterbalance these effects in the LC-MS/MS only if analyte and IS reach the source of the mass spectrometer simultaneously (co-elution from LC) and (at least in regard to ionizability) possess identical characteristics. In general, this is sufficiently guaranteed with stable isotope-labeled internal standards [68], although this “dogma of IDMS” (IDMS, isotope dilution mass spectrometry) has been shaken by some case studies [69, 70]. Analog compounds, i.e., substances with identical parent compounds and minor changes in the substituents, such as R=ethyl instead of methyl, may also be used. Here, however, caution is always advised, especially careful validation experiments are recommended. In the case of immunosuppressant TDM, ascomycin can be recommended as a good analog internal standard for tacrolimus, while the situation is not quite as clear with cyclosporine, sirolimus and everolimus [71–73].

In spite of all the efforts with assay establishment described in the previous paragraphs and even taking into account the limitation of the method, LC-MS/MS is an valuable alternative to immunoassays. The statistics of the UK-NEQAS proficiency testing (PT) scheme (<http://www.bioanalytics.co.uk>), the largest and most significant PT scheme in immunosuppressant TDM, show that there are at least 100 operational and active LC-MS/MS installations, corresponding to 20–50% of the respective total (Figure 4). Besides the clear analytical advantages, such as

- i. measurements of all immunosuppressant drugs with one platform
- ii. substance-specific analysis results
- iii. better assay sensitivity
- iv. low inaccuracy of results
- v. high trueness of analysis,

financial considerations can be brought to bear, particularly in centers with more than 10,000 specimen/year, since after the initial purchase (cost approximately €300,000) only marginal running costs are incurred per analysis.

New analysis – platforms as seen by interlaboratory testing

As mentioned above, in Europe in addition to the PT schemes offered in Germany by DGKL (www.dgkl-rfb.de) and INSTAND (www.instandev.de) the internationally acknowledged UK-NEQAS ring trial with more than 500 participants (at least in the cyclosporine and tacrolimus

sub-scheme) is available for immunosuppressant TDM. An extraordinarily high number of challenges (12 p.a. for cyclosporine, tacrolimus and sirolimus, 6 p.a. for everolimus, 4 p.a. for MPA) and the regular inclusion of patient samples (pool samples) make the UK-NEQAS results a valuable database to critically evaluate the capability of the analysis platforms. Since in the PT evaluation the immunoassays as well as the LC-MS/MS platforms are evaluated in separate sub-groups, such comparisons can be easily performed. The trueness analysis (comparison to the weighted analyte amount in standard addition samples or comparison to substance-specific measuring LC-MS/MS group) as well as the analysis of dispersion within one group (assessment of assay inaccuracy) permit valuable conclusions on the capability of measurement systems under routine conditions.

For instance, it can be shown for sirolimus (Figure 5) that a prospected change from the MEIA to the CMIA assay will be accompanied by a drastic improvement of assay reproducibility at lower concentrations. It is known that it is possible to assess the analysis accuracy within an individual laboratory (intra-laboratory CV) from the variation coefficient of the inter-laboratory test group results (inter-laboratory CV) without the laboratory itself having to conduct such investigations [74]. According to these data derived from the inter-laboratory tests by CAP (College of American Pathologists) CV_{intra} is $\sim 0.85 \cdot CV_{inter}$.

This means that if a participant group in the inter-laboratory test shows a CV_{inter} approx. 25%–30%, the margin of error $CV = 20\%$, often recognized as LOQ, is most certainly exceeded within a single participating laboratory. In this example, this means that the functional sensitivity of the

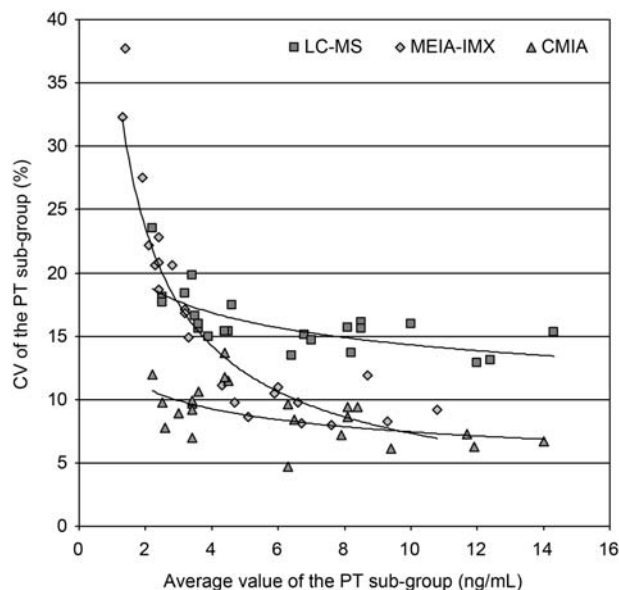


Figure 5 Relative standard deviations of the UK-NEQAS sirolimus inter-laboratory test results applied against the found group average.

For estimating the trueness of the analysis see Figure 3B and Table 1. The platform conversion from MEIA to CMIA brought a clear improvement in repeatability. Data base: 23 spike samples from 2009, group strengths: LC-MS ~ 100 , MEIA ~ 50 , CMIA ~ 25 .

MEIA lies at approx. 2–3 ng/mL, while the CMIA as well as the LC-MS-platform allow meaningful analyses far below 1 ng/mL. An analogous evaluation can be made for the inter-laboratory test results of cyclosporine, tacrolimus and everolimus (Table 1). While there are no problems in regard to the measurement precision with cyclosporine assays, some of the immunoassay platforms offered for tacrolimus, sirolimus or everolimus are not suited for conducting reproducible analyses at 3 ng/mL.

When comparing sirolimus assays it is striking that the CV of the LC-MS group is clearly above that of the CMIA group (Figure 5). Since this additional assay inaccuracy of approximately 7% is independent of the analyte concentration, this does not represent a loss of assay sensitivity at a lowered analyte concentration (as in the MEIA/CMIA comparison), but an additional contribution to the measurement uncertainty, which only manifests itself in the total picture of the inter-laboratory test. The causes for this are unclear. However, one may speculate that, especially in the area of sirolimus TDM, where many of the laboratories have used chromatographic methods a lack of alternatives [75], outdated technologies (e.g., LC-MS instead of LC-MS/MS) and heterogeneous (in-house) calibration systems (single point vs. multipoint calibrations, different ISs, etc.) are being used.

Besides the detection of deficient reproducibility, inter-laboratory test data makes it easy to see the immunologic assays' fundamental lack of trueness caused by cross reactivities. The comparison of standard addition samples with patient pool samples of similar concentrations is particularly helpful here, since besides the substances to be analyzed the latter also contain their metabolites in varying amounts and numbers. Table 1 demonstrates that for some immunoassays (e.g., sirolimus MEIA, cyclosporine CEDIA) the quantification of pure substances results in somewhat lower values than the acknowledged reference method of LC-MS/MS. Here, it is attempted with a correspondingly lower measuring calibration (negative calibration bias) to counterbalance the overestimation of the measured value occurring due to impure analyte recognition through the antibody (positive method bias) in the population mean (which is usually communicated as a stand alone value in publications) (Figure 3). This approach does not eradicate the fundamental problem of the immunoassay, i.e., the cross reactivity of metabolites with the primary antibody, which is supposed to recognize solely the drug itself. In patients only showing low metabolite concentrations these improper correction attempts may even result in immunoassay-based drug levels that lie clearly below the LC-MS/MS measured values, while patients with high metabolite concentrations continue to show values that are too high – in itself an implausible and questionable result (Figures 1A and 3B).

Outlook

In summary, it can be stated that at present there is a good selection of methods that guarantee immunosuppressant TDM. None of the methods – whether immunoassays or LC-

MS/MS – are free of analytical problems and possible error sources; both offer distinct advantages and disadvantages. Because of the high heterogeneity in laboratory structures one can expect that both solutions will continue to exist in parallel. In view of the analysis result reproducibility the new CMIA assays have laid down a benchmark, against which all other immunosuppressant TDM implementations will have to be measured. The medium-term goal of all assays must be an inter-laboratory PT group CV of no more than 7–10% in the therapeutic range. Concerning the trueness of assays, the difficulties recognized in laboratory diagnostics already decades ago [76–78] continue to remain mostly unsolved – and not only in immunosuppressant TDM [79].

Systematic measurement deviations prevent a comparison of patient level measurements beyond the limits of assays. Obviously this problem, which often cannot be realized by the clinician (because it is not communicated), impedes long-term patient management. Over the last few years the sensitivity of the assays has clearly been neglected by the manufacturers of immunoassays. Delaying the design of assays that fulfill clinical requirements for years cannot be accepted. It is also unacceptable when insufficiently validated LC-MS/MS assays are used as ‘local one-offs’. It has become obvious only recently that rule-compliant validation and participation in inter-laboratory tests are not necessarily sufficient to generate a valid assay [80]. Hence, the definition of minimum technical standards for chromatography and MS/MS are urgently needed as well as the harmonization of assay calibration must be guaranteed.

Conflicts of interest

The authors state that no conflicts of interest exist.

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