Opinion Paper

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Establishing metrological traceability for small molecule measurands in laboratory medicine

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Abstract: For molecules that can be well described metrologically in the sense of the definition of measurands, and which can also be recorded analytically as individual substances, reference measurement service traceability to a metrologically sound foundation is a necessity. The establishment of traceability chains must be initiated by National Metrology Institutes (NMIs) according to applicable standards; they are at the top and leading position in this concept. If NMIs are not in the position to take up this task, alternative approaches must be sought. Traceability initiatives established by *in vitro* device industry or academia must meet the quality standards of NMIs. Adherence to International Organization for Standardization (ISO) procedure 15193 must be a matter of course for the establishment of reference measurement procedures (RMPs). Certified reference material (CRM) characterization must be thorough, e.g., by the application of quantitative nuclear magnetic resonance measurements and by adherence to ISO 15194. Both for RMPs and CRMs Joint Committee for Traceability in Laboratory Medicine (JCTLM) listing must be the ultimate goal. Results must be shared in a transparent manner to allow other stakeholders including NMIs to reproduce and disseminate the reference measurement procedures.

Keywords: Bureau International des Poids et Mesures (BIPM); harmonization; ISO 15193; ISO 15194; ISO 17511; Joint Committee for Traceability in Laboratory Medicine (JCTLM); National Metrology Institute (NMI); quantitative nuclear magnetic resonance (qNMR); reference methods; traceability.

Introduction

Metrological traceability is the conceptual foundation of any modern instrumental measurement [1]. It is defined by the International Vocabulary of Metrology (VIM; 3rd edition, clause [2.41]) as, “Property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty” [2]. Consequently, only traceability to a higher-order reference measurement system ensures the global comparability of daily routine measurements. Since comparable laboratory results are a basic and dogmatic prerequisite for successful medical diagnostics, the limitations of comparability between laboratories were recognized very early on.

Almost 50 years ago, J. Paul Cali made the still valid statement regarding the correctness and accuracy of measurements in laboratory medicine: “Somewhere in the transition from the widely used manual methods of yesterday to the extensive use of automated methods of today, accuracy as the predominant requirement for valid results was displaced by repeatability […]. No matter what the historical reasons, there is little to be gained at this time in assigning blame. The goal in clinical chemistry today must be to reemphasize and recapture accuracy in analysis” [3]. Hence, it was a matter of course to adopt and establish the concept of metrological traceability in this complex instrumental analysis application field [4]. However, many scientific reports have since confirmed that the accuracy of the measurements, especially when different methods are used, has remained a constant challenge in laboratory medicine [5–7]. Graham White summarized the scientific

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progress made more than a decade ago: "Metrology, the science of measurement, provides laboratory medicine with a structured approach to the development and terminology of reference measurement systems which, when implemented, improve the accuracy and comparability of patients' results", and added, "The metrological approach is underpinned by the concepts of common measurement units, traceability of measured values, measurement uncertainty and commutability" [8].

Of course, the concept of traceability is valid for all International System of Units (SI units) in routine measurements, regardless of the area of application [9]. In the common understanding of measurement traceability to the highest order [10], a hierarchy of institutions is involved [11, 12]. The Bureau Internationals des Poids et Mesures (BIPM) takes the principal position in this respect. The BIPM coordinates and monitors the activities of National Metrology Institutes (NMIs), including the National Institute of Standards and Technology (NIST, USA), the National Institute of Metrology (NIM, China), the National Metrology Institute of Japan (NMIJ, Japan), and the Physikalisch-Technische Bundesanstalt (PTB, Germany) [13]. European NMIs are organized by the European Association of National Metrology Institutes (EURAMET), which recently established the European network on traceability in laboratory medicine (TraceLabMed) to set-up and coordinate research initiatives, and to improve laboratory medicine standardization.

In addition to maintaining national measurement standards, NMIs disseminate SI units nationally, oversee normative actions, provide primary reference measurement platforms, and produce primary reference materials [14]. From the formal metrological point of view, including VIM definitions and International Organization for Standardization (ISO) 17511 rulesets [15, 16], any traceability or referencing initiative by another entity cannot fulfill the role of an NMI. Reference laboratories operating under both ISO 15195 and ISO 17025 can support the diagnostic industry with reference measurement services (RMSs) based on NMI-derived primary and secondary reference standards [17, 18] (Figure 1).

However, industry and research centers that rely on accurate and traceable measurements may identify a need for standardization that may not be within the scientific or administrative purview of an NMI or reference laboratory. If so, these stakeholders will and must take actions to establish a local reference system to allow traceability of their undertaking to the highest metrological order available. For SI units like length, time, and weight, this approach is no longer necessary, as the traceability of such entities is considered solved [19, 20].

The SI unit mol *per se* is also well understood; however, the number of measurands – chemical molecules – is almost infinite in number. Even if the chemical space is reduced to those entities used as biomarkers to investigate the complex biochemical equilibrium allowing life, several hundred thousand might be targets for measurement initiatives seeking traceable and well-defined primary reference standards and reference methods. The extensive methodological complex of analytical traceability will be examined and described in the following paragraphs. A special focus will be placed on the group of small molecules, but most conclusions are independent of the chemical nature of the measurands. Possible solutions will be described that enable the stakeholders to obtain metrologically traceable analytical results more quickly than before and to correctly position new methods in the traceability chain.

Metrological traceability in laboratory medicine

Over the last century, a set of chemical entities comprising metabolites, substrates, peptides, and proteins (e.g., structural proteins and enzymes) have been thoroughly investigated in the clinical setting and understood as key components in clinical chemistry-based laboratory diagnostics. As described above, the introduction of instrumental analysis and the manufacturing of laboratory instruments by third parties established the need for inter-laboratory standardization. In contrast to other industries, scientific stakeholders in laboratory medicine swiftly established overseeing initiatives to foster measurement standardization.

The International Federation of Clinical Chemistry (IFCC) – which has a long-standing tradition for a deep understanding of the need for reference method development and utilization [21] – approached the BIPM and established a firm collaboration by founding the Joint Committee for Traceability in Laboratory Medicine (JCTLM) in 2002 [22, 23]. The main objective of the JCTLM is to consider a procedural framework for the verification of reference measurement methods, materials, and services for their compliance with international standards ISO 17511, ISO 15193, ISO 15194 and ISO 15195 [24]. Whereas ISO 17511 is the normative basis for implementing the chain of traceability into laboratory medicine, ISO 15193 serves as the normative document for setting up reference measurement procedures (RMPs) in this context. ISO 15194 describes the production and dissemination of reference material, and ISO 15195 informs on the establishment of RMSs, which may act in the traceability chain regulated by ISO 17511 [25].

JCTLM-submitted measurement methods may originate from academia as well as from metrology institutes or industry. To be deliverable to JCTLM review such methods

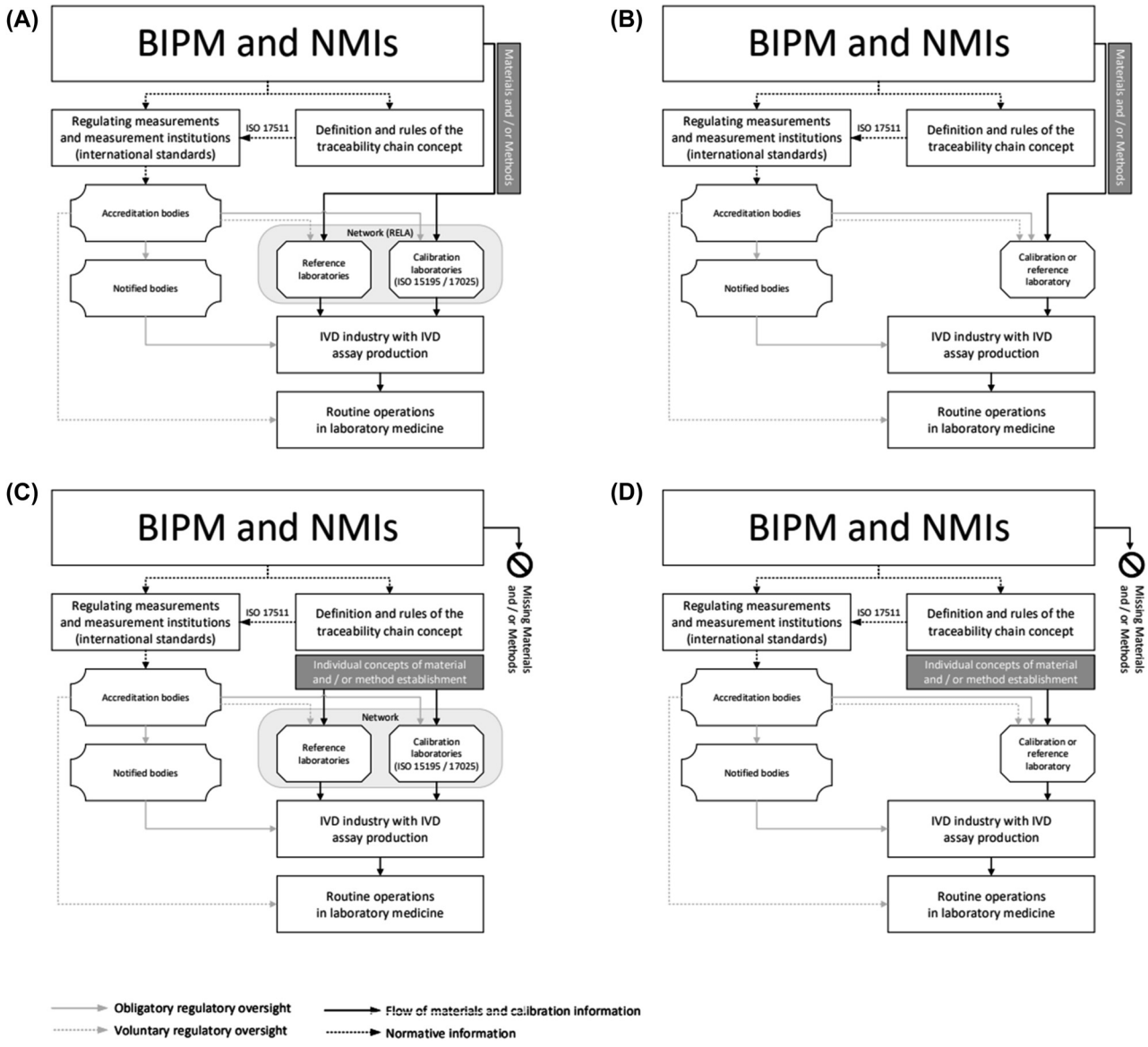

Figure 1: Illustration of four different standardization scenarios in laboratory medicine. In all cases metrological oversight is guaranteed by the BIPM and the NMIs. The concept of metrological traceability assessment is laid down in ISO 17511 and regulatory oversight is guaranteed by accreditation bodies and notified bodies. According to this setup, IVD industry relies on reference materials, reference measurement procedures and reference measurement procedures performed in reference or calibration laboratories for material value assignments to provide routine laboratories with traceable calibrator and control materials. (A) Complete and optimal metrological traceability is ensured by materials and/or methods provided/documented by BIPM and NMIs. More than one calibration and/or reference laboratory exists, the inter-laboratory comparability of the network formed is monitored in the RELA proficiency testing scheme. (B) If only one calibration or reference laboratory is providing the community with a reference measurement service, method performance cannot be assessed by ring trial-based comparisons. (C) If materials and/or methods are not provided by the BIPM and NMIs, individual traceability concepts must be followed. If more than one laboratory is performing the analysis, a laboratory comparison initiative may serve as corrective instance, which is highly recommended. (D) Currently in most cases, only one calibration/reference laboratory is available for routine calibration value assignment. If an IVD manufacturer applies this principle and communicates the reference measurement procedure transparently, e.g., by listing with the BIPM/JCTLM, then this method should be recommended for use to bring the covered measurand from situation (D) to at least scenario (C).

must have been made available to the public by peer reviewed publication. In addition to detailed experimental setup and procedure descriptions, validation and traceability data and a thorough description of the uncertainty calculation must be published. Furthermore, method transferability must be proven via a comparison study with a second measurement site utilizing the candidate measurement protocol. If an analytical entity passes the JCTLM board

examination by metrology experts, it is placed into the JCTLM database to foster dissemination in industry and academia. Reference laboratories may utilize such methods and materials to offer JCTLM-listed reference RMSs for reference material value assignment to industry and other stakeholders, e.g., external quality assurance (EQA) scheme providers [26].

The necessary worldwide comparability of analytical data to be utilized in diagnosis and therapy led to important laboratory service transforming standardization campaigns [27, 28]. International initiatives for improved standardization were launched by key organizations, such as the IFCC and World Health Organization (WHO).

The WHO successfully drove the provision of important biological reference materials for decades, especially for diagnostic entities with structural heterogeneity and limited diagnostic access due to intrinsic bioactivity [29, 30]. Early traceability initiatives were devoted to key analytes in clinical chemistry, including electrolytes, enzymes, and substrates [31–36]. At the turn of the century, further unmet standardization needs were identified for key analytes in medical decision making, such as creatinine, thyroid and steroid hormones, and cardiac markers [37–39].

Although global research efforts began in the 1970s and now span at least two scientific generations, they are still ongoing [40–42], with new and urgent tasks continually emerging [43, 44]. Within the collaboration framework of JCTLM, NMIs, and academia, a multitude of RMPs, reference materials, and RMSs have been established over the past few decades. At present, the JCTLM database holds RMPs for approximately 160 measurands, certified reference materials (CRMs) for approximately 180 entities, and approximately 120 RMSs. The database covers all analytes in laboratory medicine; metabolites, substrates, non-peptide hormones, and proteins make up more than half of all entries [45]. The overall collaboration has, therefore, been a very fruitful and sustainable endeavor that has transformed the global laboratory medicine environment [46].

Mass spectrometry-based standardization efforts

With the scientific establishment of mass spectrometry (MS) linked to high resolution separation devices, such as gas chromatography (GC-MS) and liquid chromatography-tandem MS (LC-MS/MS), hormones and other metabolites that are not enzyme substrates became possible targets for standardization. These initiatives led to an excellent traceability status of several steroidal and non-steroidal hormones (e.g., testosterone, estradiol, cortisol, thyroxine, triiodothyronine), vitamin D metabolites, and other key substrates and metabolites, such as creatinine, glucose, cholesterol, uric acid, and urea. A complete list of all available mass spectrometry based RMPs and RMSs can be found in the JCTLM database. In the field of protein analysis, the MS-fostered introduction of RMPs and reference measurement services for glycosylated hemoglobin (HbA_{1c}) was key to the development of its analytical traceability [47, 48]. The established services provided an impetus for the development of a reference method network and enabled traceable monitoring of routine measurements by means of interlaboratory comparisons [49]. These EQA schemes allow for objective assessment of the quality of routine HbA_{1c} measurement, and for the identification of successful realizations [50]. Finally, the standardization of HbA_{1c} led to the acceptance of this parameter in the primary diagnosis of diabetes mellitus [51]. However, only a very limited number of traceable protein measurements by MS have been established on the level of a JCTLM-listable RMP. This clearly indicates the technological limitations of LC-MS/MS based approaches in protein analysis, when it comes to the quality requirements of a RMP [52, 53].

Similarly, in the analysis of xenobiotics (namely, therapeutic drug monitoring [TDM] and toxicology), few developments have been recorded in recent years. Early work was devoted to the traceability of digoxin, digitoxin, and theophylline. Later, a NIST initiative resulted in a reference method and reference materials for antiepileptic drugs [54]. JCTLM-listed measurement services remain available only for digoxin, digitoxin, and theophylline, and reference materials can be obtained for a small number of other analytes. This is in clear contrast to the wide range of drugs with strong indications for TDM [55, 56] and the high diversity of Conformitè Europëenne marked *in vitro* diagnostic (IVD-CE) methods in the field.

Harmonization as an alternative to metrological traceability

In the last decade, it has become evident that the number and complexity of analytical problems, particularly in the measurement of proteins and peptides, is too large to be covered by the available resources, especially if only NMIs are involved in the establishment of a traceability chain. The results of the IFCC Scientific Division Working Group for Standardization of Thyroid Function Tests (IFCC WG-STFT)

revealed that while thyroxine and free thyroxine measurements can be made traceable [57], this concept cannot be applied to the analysis of thyroid-stimulating hormone [58], and alternative concepts must be developed [38]. Since the global goal in routine measurement is the independence of the determined measurement values from the measurement methods applied, the obvious step is to compare such methods using patient sample sets.

In 2010, the American Association of Clinical Chemistry held a conference to address this topic, and shortly thereafter the concept of harmonization was introduced to the scientific public [59]. In 2018, the IFCC working group on commutability published a series of recommendation papers to establish the framework of harmonization initiatives for measurands with several measurement systems in the market, but for which no reference materials or RMPs were available [60–63].

In agreement with ISO 17511 [64], several analyte categories can be discriminated. Measurands with available reference materials or RMPs are not targeted by harmonization efforts. In contrast, measurands lacking SI-traceable standardization might be made comparable via the use of an international harmonization protocol and could be a non-SI alternative where SI-based metrological traceability is not possible. In 2016, Drs. Vesper, Myers, and Miller stated, “Whereas standardization ensures traceability to the International System of Units, harmonization ensures traceability to a reference system agreed on by convention”, and added, “Whereas procedures and protocols for standardizing measurements are established and have been successfully applied in efforts such as the Hormones Standardization Program of the CDC [Centers for Disease Control and Prevention], harmonization activities require new, more complex procedures and approaches” [65].

This statement underpins the concept that harmonization is not an easier, but rather an alternative approach, if reference measurement systems cannot be established by conventional means [65]. This is the case if the measurand is a very heterogeneous macromolecule that is covered by more than one measurement method with different measurement principles (e.g., for parathyroid hormone, for which internationally comparable measurements beyond the manufacturer’s limits do not currently seem possible) [66].

If a measurand is definable as a singular chemical entity and measured as such (e.g., a steroid hormone, an amino acid, an antiepileptic drug, an illicit drug metabolite), SI-based metrological traceability must be sought. However, this can be problematic if no appropriate comparator method is available to harmonize. The utmost care must be taken to ensure that the VIM definition of a measurand (clause [2.3]; “the quantity intended to be measured” [2]) is adhered to, as this emphasizes that measurands are often only surrogate markers for the desired analyte to be measured. For example, in a routine measurement procedure for free thyroxine, only an equilibrium surrogate for the free thyroxine concentration actually present is measured and assigned a measured value via a stored calibration function. If the concentrations or affinities of the binding proteins change, extraordinarily large deviations in the measured value can occur [67].

Such behavior has been observed for the measurand 25-hydroxyvitamin D in serum, where biological variation in binding protein affinity leads to considerable analysis value distortions depending on ethnicity [68]. This was previously expressed by the commutability working group: “The chemical species being measured is the important consideration when selecting samples or qualifying measurement procedures for inclusion in assessing commutability of a reference material. In some cases, more than one chemical species may be measured either intentionally or because of poor selectivity of a measurement procedure” [61].

If measurands are not defined properly by the manufacturer, e.g., if one manufacturer claims to measure a certain drug, but the measurement procedure provided measured the parent drug in addition to drug metabolites present in unknown quantities and qualities in patient samples, harmonization to such an assay may be problematic if a highly specific comparator method is chosen. Vesper et al. recently stated that “a clearly defined measurand and appropriate implementation of metrological traceability with suitable reference methods and commutable reference materials are fundamental to achieving comparability of measurement results independent of time, place, and measurement procedure” [65].

In the case of TDM of immunosuppressant drugs, ill-defined measurands led to the situation that a recently issued reference material for sirolimus, namely ERM-DA111a, “was found not commutable with pooled patient samples when the […] assay was used” [69]. In this case, the measurand definition provided by the immunoassay manufacturer did not include a statement on the cross-reactivity of the diagnostic antibody with sirolimus metabolites present in patients’ blood [70]. However, the NMI producing the reference material had taken for granted the intended use statement of the manufacturer and started – in compliance with ISO 17511 – a commutability study between that immunoassay and a MS-based reference procedure. Thus, the traceability of the immunoassay to sirolimus reference materials and procedures seems impossible [71]. Harmonization approaches will also be complicated, as metabolite concentrations of drugs are known to show high inter-individual variabilities.

Consequently, whenever the analyte to be measured can be easily defined as a singular molecular entity ("type A analyte"), measurands must be unequivocally defined [72]. Cross-reactivities to metabolites or other matrix elements must be named as application limitations. If a measurement procedure is found to be highly specific, traceability to the highest metrological order by standardization must be prioritized over harmonization approaches to serve as a methodological anchor for future standardization, including harmonization.

Need for traceability chain establishment in small molecule analysis

NMIs must pay attention to new highly complex technologies that come onto the market; if poorly described in terms of measurement, these technologies can pose a high risk to patient safety if the measurement fails. At present, the moderate methodological and metrological understanding of quantitative polymerase chain reaction utilized in infectious disease, and highly parallel DNA sequencing methods (such as liquid biopsy with next-generation sequencing technologies in tumor diagnostics), necessitates such consideration [73]. Hence, analytes whose measurement quality is understood as "assured" are often lacking attention. This is especially true in the field of TDM or toxicology, where the accuracy and inter-laboratory comparability of measurements are considered solved.

However, a look at the past two decades shows that it is not self-evident that TDM and toxicology service operations offer identical and accurate measurements. Insufficient specificity of diagnostic antibodies (e.g., the problem of cross-reactivity), inadequate sample preparation, and a lack of accuracy/traceability of calibration materials are the stumbling blocks in the successful application of TDM. These problems are not historical and exist today, as demonstrated by the measurement of immunosuppressants in whole blood from transplant patients.

Transplant patients may be exposed to very high health risks in the event of treatment failure. Such risks are not confined to the local use of an LDT, since IVD-CE marked assays are usually commercialized worldwide. If diagnostic antibodies are used, insufficient separation from patient antibodies might lead to interferences leading to patient harm or death – independent from age or transplant type [74–76]. MS-based assays are also not free from errors; it has previously been shown that, for at least two immunosuppressive drugs, sufficient chromatographic separation of drug metabolites is essential if accurate testing results are to be delivered [77].

Furthermore, proficiency testing has demonstrated that MS assay results show a higher inter-laboratory residue scatter than modern ligand binding assays distributed by the IVD industry. This may be due to the more stringent industrial standardization of global/national commercial products, which is less likely to be achieved during local production of laboratory developed tests (LDTs). It can be assumed that the increased inaccuracy between laboratories is at least partly due to the heterogeneity of the calibration. This leads to individual laboratory-specific bias contributions, which are perceptible in the laboratory collective as increased dispersion of results [56, 78].

It must be expected that similar measurement deviations exist in drug classes that have not been subjected to such an in-depth analysis as immunosuppressants. Given the widespread use of these drug classes (e.g., antiepileptics, neuroleptics) and the risk-based commitment to TDM in many cases [55], patient care and well-being are expected to benefit from standardization of the measurement platforms used. Consequently, there is an obligation to establish traceability chains in TDM.

Similar statements can be made for other substance groups. For steroidal hormones, only some of these clinically important biomarker analytes have been made traceable with sufficient quality [79–81]. Even so, a recent investigation of proficiency testing data utilizing native serum samples demonstrated that, for ligand binding based routine assays, inter-assay bias contributions are remarkable and do lead to consequences in clinical decision making [82]. Although thyroid hormone standardization was successfully investigated for more than two decades [57, 58], ligand binding assay realizations from major IVD industry stakeholders show remarkable quantitative differences, with the potential to lead to misdiagnosis [83]. Serum metanephrines utilized as markers in adrenal gland tumor diagnosis are only understood as comparable in principle, and lack sound metrological standardization [84, 85]. In the structurally heterogeneous substance class of vitamins, apart from the prohormone 25-hydroxyvitamin D [86], not a single analyte has been made traceable to the highest order. The same applies to endogenous metabolites and substrates.

Of course, it is possible to compensate for the problem of systematic measurement error by adjusting the comparison intervals (reference ranges, therapeutic ranges) and decision limits for the measurement. However, it must not be overlooked that this leads to risks that cannot be tolerated. It is often common clinical practice in dealing with analysis data that only the numerical values are communicated, but not units or comparison intervals. This

expresses the fact that standardized measurement procedures and uniform measurement units are basically assumed by the clinician. This is also expressed in clinical decision limits, which are usually made for a clinically relevant parameter independent of its realization in a routine assay. If such a decision limit meets non-standardized measurement systems, clinical decision-making is significantly more difficult [87–89].

Traceability chain establishment by non-NMI stakeholders

When considering the resource limitations of NMIs alongside the diagnostic need for globally comparable routine measurements to make patient care as safe and efficient as possible, an alternative approach to the NMI-based method for establishing a valid traceability chain must be sought. Such an approach can be understood as an intermediate step toward "full traceability with NMI participation", to enable the rapid availability of reference standards and reference methods for the largest possible number of analytes. This is expected by the legislator (e.g., via the European IVD Regulation [IVDR]) and provides an opportunity for NMIs to prioritize their workload. For example, a strategy of supplementing only some of the "industrial" methods with reference methods of the highest metrological order or providing important comparative measurements can be envisioned. In this context, explicit reference must be made to the IFCC External Quality assessment scheme for Reference Laboratories in Laboratory Medicine (IFCC RELA EQAS), which provides this service independently of NMIs [90].

For successful implementation of this method, adherence to state-of-the-art approaches regarding method design and validation, and instrument qualification must be granted. CRMs which fit the intended use must be applied if available from an NMI. A given CRM is supported by documentation containing source of material, measurement results, and complete metrological traceability to SI units. If another material is used, the provider of the reference measurement platform is advised to determine the absolute content and stability, including a measurement uncertainty statement supported by either quantitative nuclear magnetic resonance (qNMR) analysis or a mass balance approach [91–93]. The developed reference methods must meet the formal ISO reference standard requirements that NMIs are required to apply.

Measurement uncertainty must be determined according to the Guide to the Expression of Uncertainty in Measurement (GUM) [94, 95]. Three main steps need to be considered: (1) uncertainty estimation of the chosen primary reference material, (2) uncertainty estimation of the preparation of calibrator materials, and (3) estimation of the uncertainty of the established reference procedure (LC-MS/MS). It is advisable to calculate the uncertainty term associated with the calibration with a bottom-up approach [96]. At the minimum, uncertainty components associated with the purity of the reference material, weighing, preparation of stock solution and dilution, preparation of spike solution, and preparation of matrix-based calibrator level must be considered, and an error propagation approach must be chosen. Typically, if an ultra-micro balance is used, the dominant uncertainty contributions are associated with pipetting; if the balance is less sophisticated, the dominant uncertainty is weighing.

The estimate of uncertainty of the RMP is derived from a precision experiment (type A uncertainty) and considers sample preparation steps, e.g., sample preparation of calibrators, preparation of internal standard, preparation of samples, measurement of calibrators, generation of the calibration curve, and measurement and evaluation of sample results. It is advisable to perform a multi-day validation experiment to allow the assessment of variability components such as between-injection variability, between-preparation variability, between-calibration variability, and between-day variability with an analysis of variance-based variance-components analysis. Consequently, the total measurement uncertainty of the whole approach (for a single measurement) can be estimated as a linear combination of the uncertainty of calibrator preparation and calculated uncertainty of the precision experiment.

All methods designed, validated, and put into use must be made accessible to the scientific public in a transparent manner, such that independent re-evaluation and dissemination is easily possible. Publications must be peer reviewed and should be accompanied by supplementary materials with detailed operational instructions. In addition, reference measurement platform candidates must be submitted to the JCTLM for ISO 15193 review. Only after JCTLM have listed a measurement platform can it be understood as reference measurement method and used as a scientific basis for service implementation.

Allowable total error of the reference method – treatment of measurement uncertainty

Currently, it is not clearly defined what level of total error is allowed in a reference method. Nonetheless, it is clear that

due to the propagation of measurement uncertainties, methods which have a priority position in the traceability chain must have a much lower total error compared with the methods in routine use. Therefore, an acceptable measurement error for each routine measurement must be determined. There are different approaches, but there is a consensus that the analytical error should be so small that the deflection of biomarker measurement results from the expected equilibrium of healthy individuals is not or is only slightly influenced by the measurement itself. The well-established model of Callum Fraser, and other models derived thereof, allows targets to be set for routine measurements based on biological variability data [97]. Reference methods must meet tighter targets; the desired factor ranges from one half [98, 99] to one third [100] of the total error allowance of the routine method. This must be done to account for the error propagation in the multi-stage traceability chain. Of course, conservative assumptions are made for the error magnitudes to minimize the risk of error underestimation.

In addition to the biological variability data, which are stored in a database of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) and available to the public [101], routine methods must generally also meet the quality requirements of regulatory authorities [102, 103]. Moreover, the fulfillment of inter-laboratory comparisons is often linked to quality requirements, which follow a total error concept (i.e., measurement uncertainty). In cases where biological variability data are not available, and if no additional guidance from the authorities is provided, reference methods must reflect the needs of the current state of the art, e.g., by the analysis of proficiency result data [56, 104]. This also applies to reference methods in TDM, because the therapeutic target ranges cannot necessarily be understood as reference ranges. Pharmacokinetic models can be used here, or simpler assumptions can be implemented (e.g., that the measurement uncertainty of a routine method should only cover a certain proportion of a target range) [105].

We consider that the measurement uncertainty of any routine method can be calculated according to the above principles. To design the measurement uncertainty specifications for a reference method from these data, the conservative approach would be to allow only one third of the total error of the routine method. The total error allowance of a routine method includes the bias term. For comparison with the expanded uncertainty of a reference measurement, the expansion factor must be considered, and, if more than one measurement has been performed, the degrees of freedom associated with the comparator measurement.

Discussion

There is no reason not to strive for traceability to a metrologically sound foundation (i.e., to materials and methods of the highest order) for molecules that can be well described per the definition of measurands and which can also be recorded analytically as individual substances. A necessity to take the path of harmonization is of secondary importance and may only be considered where no analyte-specific measurement methods are known or can be used. This basic idea is also reflected in legislative requirements, e.g., as described in the IVDR, which requires the traceability of the IVDs used.

The establishment of traceability chains must be initiated by NMIs according to applicable standards; they take the foremost leadership position in this concept. Over the past few decades, NMIs have followed this mandate wherever possible; however, the need for traceability concepts for individual analytes far exceeds the production capacity of both NMIs and downstream reference laboratories. Both the IVD industry, and also the local producers of LDTs in the fields of TDM, toxicology, endocrinology, and molecular diagnostics, are frequently confronted with the situation whereby the request of the regulating entities to demonstrate traceability cannot be met or can only be met to a limited extent.

Thus, it is necessary to establish alternative approaches to traceability. However, these can only be understood as a supplement to the existing regulations and must at least comply with the standards specified in the relevant ISO standards in terms of both basic concepts and execution. In addition to local establishment initiatives within LDTs, IVD manufacturers in particular are in a position to implement and maintain such an alternative approach. There is a great economic advantage in undertaking such an effort for an industrially manufactured IVD with appropriate market penetration. Thus, many more laboratory service providers can benefit from the traceability initiative than if each were to use individually developed LDTs. The market, down to the individual patient, must be able to expect from such an initiative that the operational design and execution of the reference measurements will be at a level not significantly inferior to NMIs or reference laboratories. Adherence to ISO 15193 must be a matter of course for such initiatives, and state-of-the-art analytical technology must be used. The calculations of the measurement uncertainties must also comply with internationally established regulations, which must extend beyond laboratory medicine and be recognized (e.g., by application of the GUM). By carefully checking the identity and purity of the reference materials, any reference

method initiative must ensure that bias contributions from the calibration are minimized. Here, the use of qNMR is recommended. This analytical approach has the capacity to check the identity of the material, to detect organic impurities and degradation products, and to perform a content determination with a single and destruction free analytical method making it superior to the mass balance approach. For the modern analysis service provider, it must go without saying that a high degree of transparency must be ensured in the communication of reference methods. In the case of reference methods, this is guaranteed by the JCTLM listing process for industry-based reference methods, as this requires both the existence of a peer-reviewed publication and the compliance of the submitted method with the basic requirements of ISO 15193.

We are convinced that RMPs meeting these requirements represent a valuable contribution to the quality assurance of laboratory analysis and give NMIs the opportunity to test them in theory and practice and, if necessary, to include them in their own portfolio. Since ISO 17511 requires that reference methods must prove that they are also suitable for measuring patient samples, such industrial reference methods with suitable throughput ensure that, in the event of unclear analysis results from routine methods, a measurement platform is available to investigate such deviations.

Summary

Generally, NMIs are tasked with establishing traceability chains. If NMIs cannot perform this task, *in vitro* diagnostic device industry and academia must be able to provide alternative solutions. Such initiatives must meet the quality standards of NMIs or calibration laboratories, with ISO 15193 adherence of the established RMPs. If reference materials are not available, materials used as such must be characterized thoroughly (e.g., through quantitative nuclear magnetic resonance measurements). Any production of reference materials must follow ISO 15194. Both RMPs and reference materials must be presented to the public in a transparent manner to permit reproduction and dissemination. Consequently, we understand JCTLM listing of materials and methods as a mandatory step.

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