

Traceability and standardization of immunoassays: A major challenge

H.A. Morris *

*School of Medicine, University of Adelaide, Hanson Institute, Institute of Medical and Veterinary Science,
P.O. Box 14 Rundle Mall, 5000 Adelaide, South Australia, Australia*

Received 18 April 2008; accepted 11 September 2008

Abstract

The clinical utility of immunoassay results is dependent on precision and trueness for the application of internationally agreed clinical protocols and common reference intervals and decision limits. The application of metrological principles to achieve traceability and standardization for these assays is being pursued to this end. Comprehensive measurement systems are available for a number of the total serum hapten assays routinely measured by immunoassays while current research is investigating the appropriateness of developing defined systems for the measurement of “free” hormone levels. The standardization or harmonization of assays for heterogeneous polypeptide hormones is also another area of research for these assays.

© 2008 The Canadian Society of Clinical Chemists. Published by Elsevier Inc. All rights reserved.

Keywords: Traceability; Standardization; Immunoassay; Hormone

Introduction

Immunoassays are commonly used in clinical chemistry to assist with the diagnosis, prognosis and monitoring of patients particularly in the context of analytes present in biological fluids at what are considered very low concentrations [1]. The reliability of clinical laboratory results is dependent on precision and trueness where trueness is of particular importance for the comparability of results between laboratories and clinical centers necessary for the use of internationally agreed clinical protocols and common reference intervals and decision limits. The concept of trueness has a considerable history in clinical chemistry in general and in immunoassay in particular. This work arose initially from the World Health Organization and their work in the preparation of International Standards particularly for peptide hormone assays [2]. These preparations were standardized against a biological response using bioassays and their concentration was expressed as a biological activity in International Units (IU). Thus as preparative techniques and purity of the preparations improved the mass of peptide per IU decreased. During the last 10 to 15 years considerable advances

have been made with technological, theoretical and organizational aspects of this field [3]. Thus we are currently in a period during which considerable progress is being made with the application of the principles of metrology to achieve traceability and standardization for immunoassays for specific measurands [4].

Why use immunoassays?

Immunoassays were quickly adopted in clinical laboratory testing because they provided a unique technology for assaying numerous analytes in complex biological fluids with adequate specificity and sensitivity for clinical use [1]. Immunoassays are capable of assaying analytes present in blood serum at levels between 10^{-12} to 10^{-6} M in the presence of substances to which they share a considerable degree of homology and may be present in concentrations of the order of 10^{-3} M. For example the steroid hormone cortisol is often measured by immunoassay at levels in blood serum of approximately 10^{-7} M in the presence of numerous steroid hormones of similar structure and at similar concentrations [5]. Probably most important in this context is the presence of cholesterol, from which all steroid hormones are derived and which forms their backbone structure. Cholesterol is present in blood serum at concentrations slightly above 10^{-3} M that is a 10,000 M excess.

* Fax: +618 822 23035.

E-mail address: howard.morris@imvs.sa.gov.au.

These properties make immunoassays such an important part of the clinical laboratory armamentarium but increase the difficulty to establish their traceability and standardization.

The fundamental principle of immunoassay is the presence of a binding agent, most often an antibody protein. The conditions for the antibody and its specific ligand reaching equilibrium with the antibody–ligand complex are governed by the law of mass action [1]. The ligand may exist as a small molecular weight substance, also known as a hapten, or a large molecular weight substance such as a protein. Consequently any substance present in the biological fluid that disturbs this equilibrium reaction can effect the quantification by immunoassay. These interferences are known as matrix effects and are a major confounding factor for the introduction of traceability and standardization in immunoassays. The automation of immunoassays and the incorporation of a wide range of these assays onto a single platform processing some 100's of assays per hour have provided clinical laboratories with considerable productivity advantages. However this development has had to accommodate a wide range of specific binding agents each with their specific binding properties and therefore different requirements for reaching equilibrium. Furthermore in the drive to optimize assay turn-around-times most of these assays have been modified such that they do not reach equilibrium [6]. Such a modification has variable effects on the quality of the assay depending on the properties of the binding agent. Thus it is likely that the quality of many immunoassays has deteriorated when included on such automated platforms particularly through increased interference by matrix effects and the requirement for increased limits of detection.

Over the recent period major technological developments have been achieved for the assay of haptens in blood serum with similar sensitivity and higher specificity than immunoassays. These assays often utilize high performance chromatography combined with mass spectrometry for detection of highly specific molecular structures [7]. Currently the major limitation of these assays is their reduced throughput of specimens compared with other assay platforms, which is inefficient for the larger clinical laboratories. As well they require specialist technologists to operate the instrumentation and their introduction to the laboratory increases the number of assay platform requirement increased resources for maintenance and quality assurance. These assays lend themselves very well to traceability and standardization and this property is likely to lead to their increasing use in clinical laboratories particularly if the throughput of specimens can be increased.

Standardization and traceability of hapten assays

As discussed in detail elsewhere in these proceedings, the key element to the establishment of traceability of measurements is a comprehensive measurement system. The essential components of such a system are an unequivocally defined analyte, a system of reference materials and reference measurement procedures [4]. Haptens are substances that because of their relatively small molecular weights are unable to initiate an immune response unless conjugated to a high molecular weight carrier substance

such as a protein. The antibody elicited in the process retains the ability to recognize and bind the small molecular weight substance. Consequently these substances have been incorporated into immunoassay platforms [7]. Examples of these substances that are important in the clinical laboratory include the steroid and thyroid hormones. Because of their relatively small molecular weight their structures have been unequivocally identified and identical materials have been prepared using synthetic chemical techniques. Thus highly purified materials are available as reference materials. Furthermore these substances have been most amenable to assay by highly specific technologies such as isotope dilution-gas chromatography with mass spectrometry detection. This technology has all the properties required for a reference measurement procedure.

Thus reference measurement systems for a number of hapten assays have been accomplished such as for cortisol [7], and this work is continuing for a number of other similar measurands [8]. The availability of highly purified material allows gravimetry to be used as the primary reference measurement procedure for assigning the values of the primary calibrator. Many such primary calibrators are available from the National Institute of Standards and Technology (NIST) [9] which for cortisol is Standard Reference Material (SRM) 921 or the Institute for Reference Materials and Measurements (IRMM) which is another source of reference materials [10]. These have been catalogued by the Joint Committee for Traceability in Laboratory Medicine and a list is available on their web site (<http://www.bipm.org/en/committees/jc/jctlm/>). These reference materials are available to calibrate the secondary reference measurement system which for cortisol is isotope dilution-gas chromatography/mass spectrometry (ID-GC/MS). It is recommended that secondary reference procedures be operated in reference laboratories under defined performance conditions linked together through networks. The JCTLM provides a list of these networks and the IFCC Committee-Traceability in Laboratory Medicine supervises RELA external quality assessment programs.

Primary reference materials are prepared in a matrix-free format for calibration of a matrix-independent secondary reference procedure. Thus the calibration of a manufacturer's working calibrator must contend with the matrix effects of the manufacturer's standing measurement procedure or routine measurement procedure as the aim is to provide traceability of the results from the routine procedure on clinical samples to the primary calibrator and primary reference measurement procedure. One method used to account for matrix effects is to prepare a series of samples in the matrix of the routine method, which in the case of the routine clinical measurement of cortisol is blood serum. This series of calibrators made up of serum samples containing cortisol can then be assayed by the secondary reference procedure to assign the "true" value and can then be assayed by the routine procedure. This method is known as the "split-sample measurement design" [4]. The relationship between the two series of values can be analyzed and used to account for the matrix effect of serum in the routine procedure. It must be recognized that a valid calibration by this method is dependent on obtaining a linear relationship between

the two sets of values across the whole range using samples from the variety of specimens submitted for routine measurements. If the routine procedure is imprecise or is subject to different interferences by different types of specimens (for example if male and female samples interfered to different levels) then this method is unsuitable.

Traceability and standardization of the biologically active fraction of haptens — the ‘free’ fraction

Each of the steroid and thyroid hormones as well as many other analytes in the plasma fraction of blood exist in at least two forms, a bound form, often bound to protein, and a non-bound or ‘free’ form [1]. The sum of these fractions is measured as the total plasma level of such substances. There is much evidence to indicate that it is the ‘free’ form that is biologically active and clinicians in many parts of the world request the ‘free’ level from clinical laboratories. This practice is best exemplified with regard to the thyroid hormones where free thyroxine (fT4) and free liothyronine (fT3) are the requested analytes for clinicians requiring information on thyroid function in many parts of the world [1]. Other non-hormone substances also fall into this category, most notably calcium where it is the ionized calcium that is the biologically active fraction.

The ‘free’ fraction exists in equilibrium with specific and non-specific binding agents. For example thyroxine binds with high affinity and low binding number to thyroid binding globulin and with low affinity and high binding number to serum albumin [1]. The measurement of ‘free’ fractions poses a number of significant problems to meet the principles of metrology. Perhaps the most important is the property that any separation of the ‘free’ fraction from the total amount in serum will disturb the equilibrium between the substance and its binding agents particularly the specific binding agents because of their property of high affinity binding. Such a disturbance will disrupt the procedure to quantify the true level of the ‘free’ fraction. This effect maybe considered akin to Eisenberg’s Uncertainty Principle [11]. One approach currently being assessed for the standardization of fT4 assays is to establish a well-defined procedure for separating the free fraction from the bound in a rapid and efficient manner using either equilibrium dialysis or ultrafiltration [12]. Currently this project is in its early stages but the results, if positive are likely to have important ramifications for the metrological standardization of a number of clinically important assays.

Traceability and standardization of peptide hormone immunoassays

At least two major issues face the application of metrological principles for the relatively large molecular weight peptide hormones. Firstly their large molecular weight currently precludes the total molecule from assays using the highest levels of analytical technique such as high performance liquid chromatography with mass spectrometry detection. Secondly these hormones very often exist in multiple forms or isotypes for which, with our current knowledge, it is impossible to

identify which isotype exerts the biological activity. This means that the assay must measure a family of peptides in order to correctly provide a measure of the biological activity in the form of a measurement of peptide mass, which is the most efficient protocol for routine clinical assays.

Two approaches have been used to resolve these problems. Firstly the aim is to identify the biologically active element and reduce it to a substance of low molecular weight that can be prepared by synthetic chemistry technologies to an adequate purity and is available as a primary reference material. Furthermore such a substance is amenable to analysis by the highest level of analytical technology suitable for a primary reference measurement procedure. If these goals can be achieved then the measurand has the necessary components for a reference measurement system suitable to provide traceability for routine clinical assays.

The best example of success with this approach has been the work with the glycated hemoglobin (Hb) form HbA_{1c} for the monitoring of diabetes mellitus [13]. The measurement of glycated hemoglobins poses many metrological issues. Firstly they are expressed as a percentage of hemoglobin and therefore two analytes are required to be defined. Secondly glycation of hemoglobin is a complex process generating numerous forms because glycation occurs at different rates at different sites. The original separation technologies upon which the nomenclature was based were poor such that the composition of the HbA_{1c} fraction varied dependent on the separation technique [14]. The clinical impetus for harmonizing the HbA_{1c} assay heightened following two landmark clinical trials where its usefulness for monitoring diabetes mellitus and identifying those patients at highest risk of developing adverse side-effects was demonstrated. In the mid 1999’s a pragmatic approach to this project was adopted [14]. This involved standardizing a chromatography system that defined the HbA_{1c} fraction. A limited network of laboratories was established to provide a service to HbA_{1c} assay manufacturers for standardizing their routine assays marketed to clinical laboratories. Thus routine assays were harmonized by this reference measurement system.

However the limitations of this pragmatic approach quickly became evident when the defined chromatography system could not be transferred to other laboratories or even its maintenance could not be guaranteed over the longer term because the technology of the system was outdated. Thus a system based on metrological principles was required that could meet the clinical requirements of traceability and standardization for HbA_{1c} measurement over the long term. A Working Group of the IFCC took forward the task of defining both analytes, HbA_{1c} and HbA₀ [14]. These definitions were based on an understanding of the physical chemistry of the peptide chain that the amino-terminal amine group was the most active with regard to adduct formation such as the glycation reaction. Thus the analytes were defined as the amino-terminal peptide obtained from a tryptic digest of hemoglobin extracted from human blood. For HbA₀ this peptide is a hexapeptide of known sequence while for HbA_{1c} it is this same hexapeptide with the addition of 1-deoxyfructose to the amino-terminal amine group. These analytes can be prepared to sufficient purity such that

gravimetry is a suitable primary reference procedure to prepare primary reference materials. Secondary reference measurement procedures have been approved utilizing tryptic digestion of whole blood followed by quantification by high performance liquid chromatography with mass spectrometry detection or capillary electrophoresis. An approved reference measurement system is in operation including a network of reference laboratories which has validated the procedure over the mid-term [13]. An important routine assay for HbA_{1c} utilizes immunoassay where the binding agent is an antibody specific to the glycated-hexapeptide and therefore this reference measurement system has been an essential development in the traceability and standardization of this assay.

This strategy is very attractive because it can be fully based on metrology principles and thus is being investigated in a number of other assays including carbohydrate-deficient transferrin [15]. However this strategy requires a significant amount of basic knowledge of the structure of the large molecular weight substance with particular regard to the site conferring biological activity or defining the biologically significant element. This level of knowledge is unavailable for most of such substances at this time and it is possible that biological activity or the essential element will not be limited to a specific site for many of them. Thus other strategies are essential for these substances.

A second approach to traceability and standardization in immunoassay has focused on assays for heterogeneous antigens or families of closely related isoforms. This strategy is based on the principle of harmonization of these assays and has a relatively long history beginning in the first half of the 20th century with International Standards prepared under the auspices of World Health Organization (WHO). These were standardized by bioassays and later used to varying levels of success to harmonize immunoassays. An example of success with this strategy is the work conducted on human chorionic gonadotropin (hCG) by the IFCC Working Group [16]. The structure of hCG is complex comprising of a highly heterogeneous family of polypeptides which have a high degree of carbohydrate fine structure. The intact molecule comprises of two noncovalently linked polypeptide chains (known as α - and β -chains) with heterogeneous carbohydrate moieties. In biological samples intact hCG is always present along with dissociated (i.e. free subunits) or degraded hCG-related polypeptides (nicked forms). The complete elucidation of this structure was not obtained until the 90's.

Immunoassays for gonadotropins were developed in the 1969's and were standardized against the WHO's preparations [2]. It was apparent by the 1989's that the lack of comparability between the routine clinical assays was not contributing to optimal patient care [3]. From a theoretical view it is clear that one problem with this approach was that the IS preparations are calibrated in terms of biological activity whereas immunoassays are measuring the mass of immunoreactive substances [4]. When dealing with such a heterogeneous family as hCG it is clearly likely that some material present in the biological fluid will be immunoreactive without being biologically active. Thus as the project developed it became apparent that at least two

issues had to be resolved before standardization and traceability of this assay could be achieved. Firstly the structure of each of the members of the hCG family needed to be accurately defined and purified preparations of each be made available. Secondly the cross-reactivity of each of the antibodies used in the routine clinical immunoassays needed to be assessed against each of the members of the family.

Each of these tasks has been completed [17]. Standards of hCG and its most important variants including the β - and α -subunits, the core fragment of hCG β and two partially cleaved (or nicked) forms are now available from WHO as the 1st Research Reagents (1st RRs). There are some 100 commercial hCG immunoassays available on the market [18]. When these assays were assessed with serum samples their maximum interassay variability was reduced to 27% when the results were calculated on the basis of the 1st RRs compared to 40 to 60% with the standards supplied by the manufacturers. Thus it was apparent that the use of impure calibrators is an important contributor to the variation of results between assays. Amongst these commercial assays any of seven antibody combinations can be employed. The variation in the specificity of these antibodies for binding to the different forms comprising the hCG family was found to contribute to greater variation of assay results than the use of impure calibrators.

This work on standardization of hCG assays has lead not only to the characterization and purification of the most common forms of the hCG family of glycoproteins but has developed a nomenclature along with calibration of each preparation in molar terms [17]. These developments have also permitted the mapping of the epitopes for each of the antibodies in commercial use. The WG has recommended that the 1st RRs for hCG and hCG β be used to recalibrate existing assays for these isoforms and that conversion factors for the reporting of existing assays in mol/L be established.

Similar developments have been made or are currently under development with assays for other analytes including growth hormone and insulin [8]. Major success has been achieved with improving the commutability of serum prostate specific antigen (PSA) assays through the introduction of an international standard comprising purified 90% PSA complexed to α_1 -antichymotrypsin (PSA-ACT) and 10% free-PSA [19]. In most instances there was little change in the absolute values achieved for total PSA in serum samples. However the variation between the different assays used in routine clinical laboratories was reduced by approximately 50% [20].

These types of projects provide a very useful outcome of improving comparability between assays through harmonization; however they do not meet the stringent requirements of traceability and standardization of these assays. Currently the only strategy for these types of substances which can meet the metrological principles of a reference measurement system, including a matrix-independent instrumental analytical method such as mass spectrometry, is to identify a surrogate analyte as outlined above for HbA_{1c}. However consideration must be given to the fact that because of the biological complexity of many of these analytes such as the example of the hCG family, will it ever be feasible to define such an analyte? This then raises the

question as to the suitability of selecting an immunoassay reference method based on a reference antibody with well-defined epitope specificity. This approach would require the definition of a standard method as has been achieved for enzymes and is being assessed for 'free' hormone assays [12,21].

There is still a very large amount of theoretical and practical development work required in this area. It is clear that to apply any of these methods will require considerable laboratory-based fundamental research and therefore expense to identify the structural and biological properties of each of the measurands as well as to develop methods for the large scale preparation of significant amounts of highly purified material required to establish reference measurement systems to establish traceability and standardization of assays. If the standard method strategy is adopted then consideration must be given to the impact that this may have on halting or at least stifling technological development for each assay. The development of the standard method should include consideration that technical developments are likely to occur to improve or even completely change the assay. It cannot be over emphasized that a major benefit of the application of the principles of metrology is to reduce the likelihood that technological developments in routine clinical laboratory assays will require changes in their reference measurement system.

References

- [1] Wild D, editor. The immunoassay handbook. 3rd ed. Amsterdam: Elsevier; 2005.
- [2] Storrer PL, Dixon H, Bangham DR. The first international standard for human urinary FSH and for human urinary LH (ICSH) for bioassay. *Acta Endocrinol (Copenh)* 1976;83:700–10.
- [3] Sturgeon CM, Ellis AR. Standardization of FSH, LH, and hCG — current position and future prospects. *Mol Cell Endocrinol* 2007;260–2:301–9.
- [4] Thienpont LM, Uytendaele K, De Leenheer AP. Reference measurement systems in clinical chemistry. *Clin Chim Acta* 2002;323:73–87.
- [5] Lantto O, Björkhem I, Blomstrand R, Kallner A. Interlaboratory evaluation of 4 RIA kits for determination of plasma cortisol: influence of matrix in calibration standards. *Clin Chem* 1980;26:1899–902.
- [6] Sikaris K, McLachlan RI, Kaslauskas R, de Kretser D, Holden CA, Handelsman DJ. Reproductive hormone reference intervals in healthy fertile men: evaluation of automated platform assays. *J Clin Endocrinol Metab* 2005;90:5928–36.
- [7] Thienpont LM. A reference system for cortisol. *Clin Biochem* 1998;31:483–8.
- [8] <http://www.ifcc.org/> for IFCC Annual Report 2006.
- [9] <http://ts.nist.gov/measurement-services/reference-materials/index.cfm>.
- [10] <http://irmm.jrc.europa.eu/>.
- [11] http://en.wikipedia.org/wiki/Uncertainty_principle.
- [12] Thienpont LM, Beale G, Christofides ND, et al. Proposal of a candidate international conventional reference measurement procedure for free thyroxine in serum. *Clin Chem Lab Med* 2007;45:934–6.
- [13] Weycamp C, John WG, Mosca A, et al. The IFCC reference measurement system for HbA1c: a 6-year progress report. *Clin Chem* 2008 Feb;54: 240–8.
- [14] Goodall I. HbA1c standardisation destination—global IFCC standardisation. How, why, where and when—a tortuous pathway from kit manufacturers, via inter-laboratory lyophilized and whole blood comparisons to designated national comparison schemes. *Clin Biochem Rev* 2005;26:5–19.
- [15] Jeppsson JO, Arndt T, Schellenberg F, et al. Toward standardization of carbohydrate-deficient transferrin (CDT) measurements: I. Analyte definition and proposal of a candidate reference method. *Clin Chem Lab Med* 2007;45:558–62.
- [16] Stenman UH, Tiitinen A, Alfthan H, Valmu L. The classification, functions and clinical use of different isoforms of HCG. *Hum Reprod Updat* 2006;12:769–84.
- [17] Stenman UH. Standardization of assays for human chorionic gonadotropin. *Clin Chem* 2004;50:798–800.
- [18] Cole LA. Immunoassay of human chorionic gonadotropin, its free subunits and metabolites. *Clin Chem* 1997;43:2233–43.
- [19] Stamey TA, Chen Z, Prestigiacomo AF. Reference material for PSA: the IFCC standardization study. *Clin Biochem* 1998;31:473–81.
- [20] Sturgeon CM, Ellis AR. Improving the comparability of immunoassays for prostate-specific antigen (PSA): progress and problems. *Clin Chim Acta* 2007;381:85–92.
- [21] Schumann G, Aoki R, Ferrero CA, et al. IFCC primary reference procedures for the measurement of catalytic activity concentrations of enzymes at 37 degrees C. *Clin Chem Lab Med* 2006;44:1146–55.