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Scientific and Standardization Committee Communication: A Reference System Approach to Future Standardization of Laboratory Tests for Hemostasis [1, 2, 3]

A Position Paper of the Joint Committee of the IFCC Scientific Division and the ISTH Scientific and Standardization Committee

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A description of the development of a strategy for improving the standardization of coagulation tests and the rationale behind the recommendations that follow are the subject of this position paper. Such an endeavor has been occasioned by the advances in the technology for the diagnosis and therapeutic treatment of hemorrhagic and thromboembolic diseases. These and anticipated further advances make it imperative that the standardization of laboratory tests used in diagnosis and in monitoring therapeutic treatments be the 'state-of-the-art' so that the accuracy, comparability and clinical utility is of the highest order. The complexity of the hemostatic system makes achieving accuracy and comparability among these tests a substantial challenge. Another impetus for improving the comparability of the results from different laboratories throughout the world arises as a consequence of the globalization of the pharmaceutical and medical diagnostic companies that began in the 1990's and is continuing into this, the 21st century.

The assay of a component of the coagulation system presently employs a "substrate plasma" deficient in the component of interest and the measurement of the elapsed time between initiation of the clotting process and clot formation (gelation). Quantitative assessment of the activity of this component is achieved through a dose-response relationship between a dilution of a reference plasma that contains all coagulation components in their "normal concentrations and the elapsed time (clotting time) for each dilution. Many variations and modifications of this approach that have improved the specificity of these tests are in routine use today. These methods have been key to many of the biochemical advances made in the past and have been invaluable to local laboratories with expertise and understanding of hemostasis. However, coagulation tests are now being passed to contract laboratories where those responsible for performing these assays do not have the necessary background in coagulation.

Independent of the need for advancing the art of coagulation testing; the completion of the human genome project will mandate a change in the perspective that must be taken to standardize the protein products that the genome encodes. Phenotypic expression of impaired

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[1] Hemostasis is intended to be interpreted broadly and to include proteins components of coagulation, fibrinolysis and platelets.

[2] Designated C-SCT, Committee on Standardization of Coagulation Tests in the IFCC Scientific Division

[3] A decision was made by the C-SCT to exclude the two common or "global" screening tests, the PT and the aPTT and variations of these tests such as the aPTT used for heparin monitoring and in APC resistance detection from this effort. The proposals made here are not intended to apply to these tests, nor have tests related to platelet function been considered.

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protein function (dysfunction[4]), will be related directly, by reference to mutations and polymorphisms, to the underlying causes at the genetic level. The genetic defects underlying many hemorrhagic diseases have long been established and specific mutations that create some distinct phenotypes are known. The combined effects of the environment and multiple genetic factors (mutations and polymorphisms) are emerging rapidly as important risk factors for thromboembolic disease (1).

The advances in the technology for identifying genetic polymorphisms and the rapidity with which this is becoming commonplace virtually guarantees that data describing individual polymorphisms will increase dramatically. Because of these technical advances identification of many single nucleotide polymorphisms will almost certainly precede the identification of any molecular dysfunction to which the polymorphism may be related. It can be anticipated that with so much rapidly accumulating information, confusion about the relationships between the polymorphisms and their functional significance will be commonplace as well.

Recommendation: *Approaches to the Standardization of the Coagulation factors should be made which take into account the relationship between the structures and the functions of the individual proteins.*

Consequences for Coagulation Test Standardization of Relating Function to Protein and Gene Structure

The first requirement of relating protein structure and function to gene structure, directly relevant to the standardization of laboratory methods, is that a gene structure-based reference system for proteins is molecule (mole)-based.

The second requirement of the mole-based system is that reference materials will need to be single protein reference preparations, which possess consensus amino acid sequence and identical post-translational modifications as the wild type protein.

The third requirement for linking gene-structure directly to protein-function is that a quantitative description of the degree of protein dysfunction will need methods for the measurement of biological activity with a sensitivity and specificity adequate to relate gene mutation or polymorphism and protein dysfunction. Without such assays it will be unlikely that polymorphisms that are causes of protein functional impairment and risk factors for disease will be distinguishable from polymorphisms that are not. This is the underlying philosophy for adopting single substance reference materials and including reference methods as components of a system for the standardization of coagulation tests.

Recommendation: *The base units for expression of amounts of protein should be moles in future standardization efforts. Conversion of moles to "traditional" units should only be done by a metrologically sound procedure.*

[4] Dysfunction at the molecular level is conceived as altered binding affinity and/or catalytic efficiency. The terms are introduced to facilitate discussion and to provide a basis for comparing functionality of "normal" proteins with the products of mutations or polymorphisms that are quantitatively different form the "normal" molecule.

Substances Not Currently Amenable to Standardization through Rigorous Metrological Approaches

Rigorous metrological approaches that are molecule based may not be readily applied to some substances involved in hemostasis. An example is von Willebrand's factor, a multimeric protein that circulates as a family of oligomers that differ in the number of linked protomeric units. Other examples are platelet receptors and similar membrane-bound molecules. Standardization of these molecules may require more traditional "biological" approaches until such time as molecule-based approaches become practical.

Relationship of the Existing Reference Materials (Standards) and the Proposed Reference Materials for Hemostatic System Components

Previously established reference preparations, both single substance and plasma reference preparations have been used primarily to establish reference values for biological activity (potency), although not exclusively so. The potency is commonly determined by a variety of different bioassay methods and the value for the reference material assigned from the mean value of the assay results. Although these reference materials can be credited with substantial advances in the standardizaton of coagulation tests, their limitations in view of the genomics advances described above are now becoming apparent.

It is practically impossible to prepare identical pools of plasma in different laboratories, or from geographic regions in which the spectrum of the genetic polymorphisms is almost certainly variable. The discovery of Factor V^{R506Q} exemplifies this extremely well. Other sources of variability, e.g. in the amount of the reference substance because of regulatory gene variations or in the amounts of other proteins that may influence the results of assay measurements cannot be eliminated. Similarly, it is impossible to rigorously relate such arbitrary biological activity units that are determined by averaging results from different types of assays to the number of molecules present in a pool. It is also clearly impossible to create several identical plasma pools, collected at different times, from the same donors all in the same state of health, as they were when the original donation was made.

From the gene-sequence reference perspective described above, the limitations of plasma pools make them unsuitable for meeting the requirements for reference materials in the future[5].

Single substance standards (reference materials) already exist for some coagulation components that are homogeneous purified proteins and a few are recombinant proteins. The current antithrombin standard from ISTH/SSC and WHO is a single, homogeneous substance, and the t-PA standard is a recombinant protein. These are obvious candidates for the next generation of reference materials for hemostatic system components. Additional purification may be required to eliminate partially proteolyzed forms of some substances, e.g. from recombinant plasminogen activators

Recommendation*: . Further characterization of the homogeneous products should be the first step toward achieving rigorous traceability between these and future reference materials.*

[5] Plasma pools may be suitable as secondary reference materials after the values for the protein(s) of interest have been established by metrologically sound methods.

Mole-based Standardization, Biological Activity and Reference Intervals (Normal Ranges)

Assay method standardization that is based on rigorous metrological principles will produce values for the substances that are based on SI units. When a substance in a sample is measured by a reference method that is calibrated with a reference preparation for which the molecular characteristics have been established, the result CAN be expressed as mole of the substance. When the reference method is based on a biological activity of the substance, then the initial assay result is biological activity, e.g. katals for an enzyme. Using the relationship between the biological activity and the mole amount that is derived from the single substance reference preparation, the result obtained from the reference method can also be expressed in moles of substance. The accuracy of such a conversion from biological activity to moles requires the reference method be extensively characterized so that corrections can be made for the biases caused by interfering substances that might be present in the sample.

Comparison of an existing reference preparation, e.g. a plasma pool "standard" with a single substance reference preparation permits the relationship between the moles of substance defined by the reference preparation and the substance amount present in the "standard". When this is done using the reference method, the bias that may be caused by other components present in the plasma pool "standard" can be identified and correction made for them. After such comparisons have been appropriately made a relationship between the arbitrary activity units of the plasma pool "standard" and the single substance reference preparation can be defined. Following determination of such relationships, reference intervals for establishing the normal variation among healthy individuals can be expressed both in moles and in "traditional" units (percentage of the "normal pool"). As noted above, the use of analyte depleted "substrate plasmas" for obtaining specificity in coagulation time based tests creates a potential source of variability and bias even when mole-based and calibrated reference materials are used. Approaches to eliminate bias from "substrate plasma" variability will be considered in subsequent discussions of the reference method contribution to improving standardization of coagulation testing.[6]

When routine methods are calibrated against a single substance reference preparation and compared with the results obtained using a reference method, bias inherent in the routine method can be identified and a correction applied. Again, the relationship(s) between different routine methods and the metrologically established reference preparation and reference method form the basis for expression of results from different routine methods. From these comparisons between reference method and routine methods, comparability between the routinely used methods can be established and correct diagnostic conclusions to be drawn, independent of the test method or supplier.

Development of a Reference System for the Components of the Hemostatic System

The value of a reference system comprised of reference materials and reference methods was initially recognized in chemistry of simpler substances, not substances of the complexity of

[6] Requirements and procedures for validation of methods are a critical component in the development of standardization methods that can ensure better accuracy and comparability of coagulation tests. Existing knowledge from pharmacokinetics, e.g. Shah, et al. (1991), Analytical Methods Validation: Bioavailability, Bioequivalence, and Pharmacokinetic Studies. J.Pharm. Sci. **81**: 309 are very relevant.

biological macromolecules. The advances in the development of protein mass spectrometry has made it possible to apply this approach to proteins and biological systems as complex as hemostasis. The very substantial advances in the isolation of proteins from plasma in a chemically homogeneous form and the cloning and commercial production of recombinant proteins no longer exempts the standardization of coagulation proteins from metrologically sound procedures.

The necessity for and advantages of a metrologically sound reference system for achieving compatibility of assay results in clinical chemistry have been reviewed extensively with regard to analytes common in clinical chemistry (2-5). In the past, however, coagulation tests have commonly been exempted from evaluation in rigorous metrological terms because of their complexity.

Determination of the relationships between the fundamental, molecule-based units that can be related to the molecular structures as determined by the gene structures that encode for them and "traditional" 100% activity units can only be achieved through application of the established and validated methods of metrology. After the single substance reference materials have been characterized by state-of-the-art analytical techniques and by the newly developed reference methods, the determination of the relationship(s) between the "units" assigned to the previous reference materials (standards) and the mole-based values of new primary reference materials should be undertaken.

Recommendation: *In the future primary reference materials should be homogeneous protein preparations that have been extensively characterized by state-of-the-art methods. They should be available in sufficient amounts that manufacturers, reference laboratories and laboratories participating in epidemiological studies can standardize their secondary reference preparations with respect to them.*

Proteins Isolated from Plasma versus Proteins Prepared by Recombinant Technologies

As already noted, several proteins of the hemostatic system are available in homogeneous form and are the first candidates for further characterization. Moreover, a few products of recombinant technology are already adopted as standards. Recombinant Factor VIII and plasminogen activator, tissue type (t-PA) are widely used therapeutic products; Factor VII is becoming so. True chemical homogeneity is only obtainable using recombinant technologies for protein production, although by no means guaranteed for them. Because the recombinant protein will have a single, defined sequence and composition, it is the ideal substance when it can be produced and folded correctly for reference to the gene sequence from which it is determined. Post-ribosomal modifications, including proteolytic modifications must be recognized and as completely defined as the amino acid sequence.

The recombinant material is a reference substance for which constancy is primarily important. If the recombinant protein sequence is the same as the most prevalent natural sequence, the need for a conversion factor to relate the recombinant protein functional properties to those of the protein encoded by the most prevalent allele is eliminated.

Recommendation: *Whenever possible future reference materials should be recombinant products. The exon sequence selected should be from an individual with fully functional protein, to the extent that this can be known. The recombinant product must be a chemically*

homogeneous protein preparation that has been extensively characterized by state-of-the-art methods. It must be identically folded to the native protein and post-ribosomal modifications must be either so similar to the native material or the differences characterized so that any functional differences can be known and compensated. Description of the preparation of the recombinant protein should be sufficient to enable the protein to be produced in laboratories or facilities other than the facility that prepares the initial batch.

Development of Reference Methods that Explicitly Address the Multi-functional Nature of Hemostatic System Components.

Reference methods are designed to be of high accuracy and precision and to be minimally susceptible to interfering substances. To achieve these goals, the ideal reference method should employ chemically homogeneous materials for all the reactants of the reference method in addition to the specific reference material. Under these restrictive conditions the reference method for an enzyme would be the source of the values for the catalytic efficiency of the enzyme in a selected reference reaction. It is from the differences in catalytic efficiency of proteins synthesized from genes with polymorphisms and mutations that will enable the functional significance of the different species to be recognized

One of the conventional uses of reference methods is in the determination the amount of protein or enzyme present in a secondary reference material by comparison with the primary reference material. Examples are the standardization of secondary materials such as those used by test kit manufacturers in ensuring lot to lot consistency in their products (e.g. calibrators and controls). Secondary materials are also used by specialized research or testing laboratories that require traceability to international standards for the validation of the results of their testing procedures (e.g. those involved in epidemiological studies and EQAS programs). Reference material batch sizes should therefore be of sufficient size to enable calibration of secondary reference materials to be made by all legitimate users.

In addition to reference materials, conventional coagulation methods commonly employ specific, analyte-depleted “substrate plasmas”. Because of unrecognized differences between preparations of depleted ”substrate plasmas”, reference methods must address substrate plasma-related sources of bias in assay procedures.

Because a large number of the proteins of the hemostatic system are catalysts, (e.g. proteinases and cofactor proteins), a starting point for the development of reference methods for these components are the previously proposed guidelines from IFCC on reference methods for the catalytic concentrations of enzymes (5) and the report of the PGM of the Subcommittee on Fibrinolysis of the SSC (6). Reference methods for proteinase inhibitors can also be included under this grouping because they modulate the activity of proteinases and thus the measured property of the reference method reactions is the effective proteinase concentration as a function of time.

Recommendation: *The <u>first</u> in this series of reference methods should be developed for the assay of proteinases and proteinase inhibitors.*

Three characteristics of the hemostatic system that have not been encountered in the development of reference methods for other enzyme systems must be considered for hemostatic system components. These characteristics are 1) multiple structural and functional domains within individual hemostatic system proteins 2) several physiologically important substrates for

many of the proteinases and 3) the self-assembly of hemostatic system proteins into multicomponent catalysts of activation of proenzymes.

The first characteristic, that most of the individual proteins consist of several structural domains that have distinct functions in the reactions in which they participate, may require that reference methods include subsidiary methods (submethods) that are differentially sensitive to the functional properties of the various structural domains. Gla domains and Ca^{2+} binding and Gla/Ca^{2+} mediated phospholipid binding is a well-known example of one structural domain.

The second characteristic, multiple protein substrates for one proteinase, will require reference methods that include the different substrates. Each substrate may be a "submethod" of the parent reference method. Using thrombin as an example, the list of substrates might include fibrinogen, Factor XII, Factor XI, Factor VII, Factor V, Factor VIII and Protein C. Because thrombomodulin is a cofactor for thrombin activation of Protein C, it would be part of a "submethod" for thrombin in Protein C activation. However, the sub-methods are also the principal components of the reference methods for these alternative substrates in the reactions that involve them. Similar considerations are relevant to the use of "substrate plasmas" because of the potential variability in components other than the analyte removed from the "substrate plasma". Such variability might be included under matrix effects, however because of the participation of several components in the reaction complexes involved in reactions of hemostasis, these are probably better considered as reactants that are explicitly defined in the reference methods.

The alternative substrates, e.g. for the reference reaction of thrombin acting on fibrinogen, are competitive inhibitors of the reference reaction and thus are interfering substances. Consequently, the submethod data also become the basis for determining the quantitative importance of these other proteins on the thrombin/fibrinogen reference method if they contaminate the fibrinogen preparation. Other interferences would be antithrombin, heparin cofactor II and _-2 macroglobulin. By-products of the reference method development are specifications for the effects of interfering substances in secondary reference materials and kit calibrators. Obviously, this is one of the ways in which accuracy of tests is improved through specification of the interferences that can bias the assay for an individual substance. Interference will almost certainly occur as the result of other proteins and lipids in plasma and "matrix effects". These can be independently evaluated from measurement of the effects of plasma on the reference method.

When routine methods are compared with reference methods, some substances will be interferences in the routine methods that are not identified as interferences in the reference method. An example would be fibrin(ogen) degradation products that would be interferences in clotting methods that would not be interferences in chromogenic substrate-based methods.

Third, in most, if not all of the reactions of hemostasis, complexes of two or three different proteins interact to produce the physiologically relevant, fully functional reaction complexes. Two examples are 1) Xa, Va and prothrombin (plus a phospholipid surface and Ca^{2+}) in prothrombinase and 2) plasminogen activator, tissue type (t-PA), fibrin and plasminogen in plasminogen activation. Reaction complex formation, the mechanism by which rapid clotting can occur at injury sites, results in large reaction rate increases that will require special technical considerations in the development of reference methods that include cofactor proteins and

surfaces. Development of methods for cofactor proteins and activation complexes will have to address the added technical complexity that their formation produces.

Recommendation: *Considerations regarding the development of reference methods for such rapid reactions should be deferred until methods for simpler systems have been developed.*

Operational Approaches to Development of Reference Methods and Single Component Reference Preparations.

The challenges of pursuing this strategy notwithstanding, an approach based on a recombinant reference material and metrologically sound reference methods was agreed to be developed for antithrombin. Antithrombin was chosen because of the extensive and concordant data related to structure, mechanism of action, known mutations and polymorphisms and the clinical importance of antithrombin as the principal agent through which heparin anticoagulation is achieved. A working group, the "Antithrombin Working Group" will be established for this purpose. Coordination of the activities of the working group will be under the guidance of the Joint Committee on Standardization of Coagulation Tests (C-SCT). The operation of the group will be the responsibility of two co-chairs, one who is a member of the SSC Subcommittee on Plasma Coagulation Inhibitors and one from a Society that is a member of the IFCC. It was proposed that the working group include experts in protein structure, recombinant protein production, state of the art protein characterization, mechanism of action of serine proteinase inhibitors, specific antithrombin molecular genetics and individuals with specific interest in accurate methods for diagnostic test development and antithrombin-related therapeutic drug monitoring. Consultants who have experience in reference systems based on metrological principles will be recruited to advise the working group by commenting on interim reports of the group will be recruited from individuals in academia, industry and national and international regulatory agencies. In keeping with IFCC practices, representatives to the C-SCT from national clinical chemistry societies and industry members of IFCC will be invited to comment on the proposals for the antithrombin reference material and the reference methods being developed.

Significance of the Expanded Reference System Development Recommendations

The development of reference preparations and reference methods is a long-standing activity of professional organizations such as IFCC for clinical chemistry and reference preparations (standards) by the ISTH SSC and its predecessor, the ICTH for hemostasis testing. Both organizations are involved in standardization mandated by national and international treaties, commissions and regulatory agencies. The recommendations presented in this position paper create new opportunities for cooperation and sharing of expertise. However, the changes in how functional properties are to be related to protein sequences and to gene sequences is new to all. The emerging field of proteomics will address some of the challenges associated with associating gene (exon) sequences and the proteins that they encode. However, the task of distinguishing "functionally neutral" single nucleotide polymorphisms and polymorphisms that possess functional significance should be accepted by those who know this system. The joint effort by the ISTH/SSC and the IFCC will be an expanded and metrologically sound approach to standardization. Because it initiates an approach to evaluation of the i*n vitro* significance of the functional differences in proteins encoded by different alleles and polymorphic genes, it may make the two organizations leaders in addressing the anticipated confusion about the functional importance of newly identified SNPs and polymorphisms. It can be expected that the

development of new reference materials and reference methods will also aid in the development and evaluation of new therapeutics that reduce the risk of thrombosis in individuals genetically so predisposed.

Application of the approach developed in this position paper will follow in a second document in which examples of the application of metrological principles to coagulation components will be described.

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