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New initiatives in the standardization of protein measurements

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Abstract

Unlike assays for ions and small molecules plasma protein measurements depend entirely on comparison of a test sample with a calibrant, known as a reference material or standard. Owing to the innate molecular heterogeneity of plasma proteins it is often impossible to ensure that the calibrator is identical in its behaviour in measurement systems to the test sample. Despite. these fundamental limitations it is clear that internationally agreed calibrants are the only way to improve consistancy of reporting of protein values in biological fluids between methods and laboratories. Unfortunately, only international units are not subject to contention and the use of mass values which are not internationally agreed, and are re-ascribed during the life of a standard, has jeopardised the value of international calibrants. If mass values are used it must be accepted that they are, to some extent, arbitrary.

Introduction.

The measurement of the principal human serum proteins has assumed an increasing role in the detection, diagnosis and monitoring of disease and recently certain serum proteins have also been recognised as useful in the assessment of health. The quantitative measurement and qualitative assessment of a variety of serum proteins can be viewed as a biochemical biopsy that is readily accessible, can be repeatedly inspected, is relatively non-invasive, and can yield useful information much of which is not attainable by other means.

The immunoassays used for plasma protein measurement are capable of a high degree of sensitivity and specificity. They are, however, influenced by the nature of the antibody and antigen, and for ideal performance require antisera of defined specificity, a single homogeneous molecular species of analyte, and calibrators which behave in an identical fashion to the test sample in the assay system. In fact these criteria are rarely all met and compromise is necessary. No measurement system exists for individual proteins in a mixture that provides an absolute signal dependent upon the amount of analyte present. Small molecules and ions have characteristics such as molar absorbtivity which, once established, can be used to measure concentration in aqueous solutions or biological fluids. Interference may arise from contaminating molecules or molecular interactions, but an absolute measurement characteristic can be attributed to a molecule. In contrast immunoassays are entirely a matter of comparison of a test with a calibrator

A variety of reference materials for plasma proteins have been in use world-wide over the last 10-15 years (1-6). These standards have values assigned in International units and mass units by several methods against a number of different primary materials. Despite the development in the late 1960's of the World Health Organisation's Standard for Immunoglobulins in Human Serum and in the late 1970's of the Standard for 6 Human Serum Proteins, quality control surveys in Western Europe and the United States during the early 1990's showed that values for a given protein may vary by as much as 50 to 100 per cent, depending on the reference used (7). The solutions to this problem are complex, but the use of a single, international reference material by all manufacturers and laboratories has been shown to reduce the variability to a large degree.

This has been achieved by the production of a new international reference material for 14 plasma proteins which has been produced by Community Bureau of References of the Commission of the European

Communities (BCR); BCR CRM 470. The project has been managed by the Committee on Plasma Protein Standardisation of the International Federation of Clinical Chemistry, and the College of American Pathologists (CAP), the major providers of plasma protein reference materials in the USA, have adopted the standard. The material was certified by BCR as a certified reference material (CRM 470) and released jointly with the CAP in mid-1993. Values have been assigned for transthyretin (prealbumin), α1-acid glycoprotein (orosomucoid), α1-antitrypsin (α1-protease inhibitor) and transferrin against new purified protein preparations. Values for albumin, ceruloplasmin, haptoglobin, α2-macroglobulin, C3, C4, IgG, IgA, IgM and C-reactive protein have been assigned against existing international reference materials according to a rigorous protocol designed to test the appropriateness of the candidate preparation as well as the performance of the collaborating laboratories (8-12). A further project has been completed to assign values for α1-antichymotrypsin and immunoglobulin kappa and lambda light chains. The ideal hierarchy of international reference materials is shown in Fig 1.

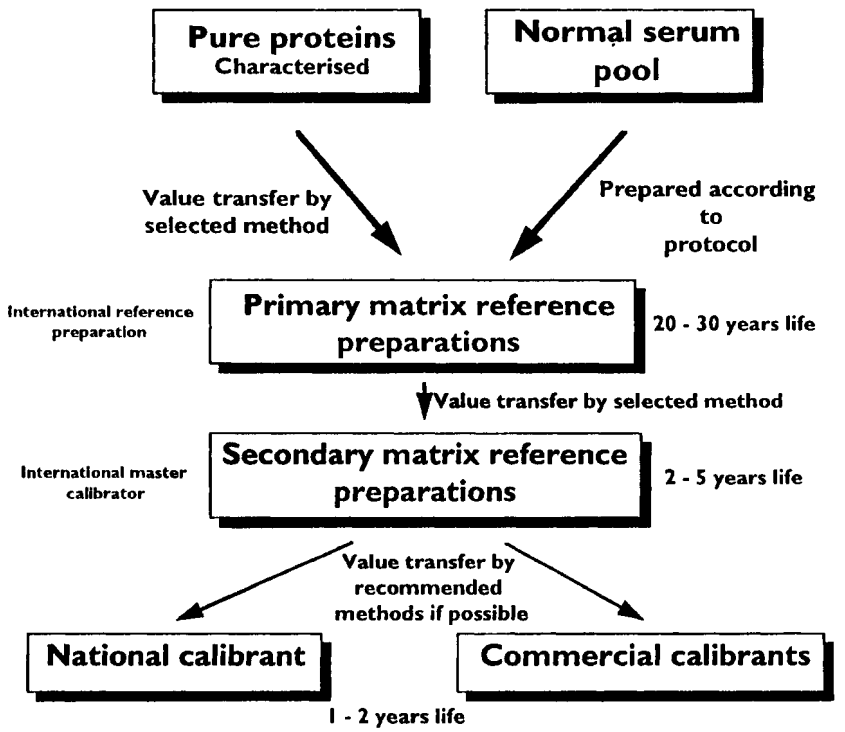

Fig 1. Classification of international, national and commercial plasma protein reference materials.

Preparation of CRM 470, the New International Reference Preparation for Proteins in Human Serum.

In most cases matrix based calibrants are desirable for all immunoassays as they are more likely to behave in a similar fashion to patient's samples comprised of the same matrix. However plasma protein calibrants may require special treatment to render them suitable for all assay techniques. Lyophilisation destabilises lipoproteins present in the serum so that the materials are turbid on reconstitution and unsuitable for immunonephelometric and immunoturbidimetric assays. Delipidation must thus be performed by methods which do not damage proteins.

Of major importance in the production of CRM 470 was the requirement for the material to behave in a similar way in assay systems to patient's serum samples. This was achieved by the use of naturally clotted serum and preservation with protease inhibitors. The proteins present within the material were thus in a similar physical state to those in fresh serum and showed no alteration on storage except in the case of C3 where it was converted to a stable form. This is desirable as proteins should be in a stable state unable to degrade further during the life of the calibrant, a potential problem with native C3..

Optical clarity, so important in modern immunonephelometric and immunoturbidimetric assay systems, was achieved by bleeding volunteers after an overnight fast, allowing spontaneous clotting of the blood in glass, rejecting any donations which were visibly turbid, and absorbing lipoproteins with microparticulate silica. This procedure was extremely effective although it caused a significant decrease in overall volume and a reduction in concentration of some proteins with an affinity for the particles, such as C4 and IgM.

The primary calibrants

Ideally, international reference materials with values assigned against purified and highly characterised proteins are desirable. Although pure proteins are rarely used beyond the calibration of the matrix reference preparation, they may be added to it to increase the concentration of individual analytes. Until the advent of recombinant proteins it would be safe to say that 'pure' proteins could not be prepared by conventional methods. A protein obtained from biological fluids always contains small amounts of contaminants which should be at a low, but defined, level. Almost all peptides and proteins in biological fluids show some degree of molecular heterogeneity and purification methods frequently subfractionate these forms. A protein for use as an immunogen or primary calibrant should ideally contain the same heterogeneity as that to be encountered in the test sample. Indeed several preparations of the same protein may be prepared comprising different molecular variants and tested for their behaviour. Pure proteins which show the same spectrum of microheterogeneity as the protein present in plasma have rarely been prepared. However, The Working Group on Plasma Protein Standardisation of the International Federation of Clinical Chemistry, undertook this task during the early 1980s for three plasma proteins; transthyretin (prealbumin), α1-acid glycoprotein (orosomucoid) and transferrin. This was achieved by using mild purification methods that do not damage the proteins and by achieving very high recoveries which are the best guarantee that subfractionation has not taken place. In practice after several years work recoveries of 85% for transferrin, 70% for orosomucoid and 55% for transthyretin were achieved. Purity assessed by electrophoretic and immunoelectrophoretic techniques showed contaminating proteins at less than 0.01%. In practice, for immunogens and calibrants, recovery has a higher priority than purity beyond this

Where pure proteins are difficult to produce and recombinant materials are available they can be, and frequently are, used as both primary and secondary reference materials. The cytokines provide an example of international reference preparations all of which are recombinant proteins. Unfortunately it is frequently not clear which gene of several possible allotypic variations has been cloned and whether or not this matters in terms of immunoreactivity. In addition, unless a mammalian expression system has been used, recombinant proteins lack carbohydrate side chains which may alter mass values attributed and immunoreactivity. Interleukin 6 (IL-6) is a good example. A recombinant interim international standard exists (13), but we now know that the IL-6 gene has several complex polymorphic systems affecting the structural gene and the 5 prime and 3 prime flanking regions. In the case of the structural gene such polymorphisms maybe expected to alter the sequence of the protein and possibly its conformation and immunogenicity, particularly for monoclonal antibodies. The importance of investigating such phenomena for the plethora of IL-6 assays appearing in the literature and on the market is obvious. Recombinant materials have not been used as reference materials for the major plasma proteins and part of this project was to asses the suitability of such materials for a large glycosylated protein, α1-antichymotrypsin

For proteins and peptides the most practical way to express concentration is in mass per volume rather than in mol per volume as molecular weight is frequently not agreed upon and may vary according to the biological state of the protein. It seems logical that dry mass should always be used as the basis for assigning value to a primary calibrant. The process of drying destroys the protein and therefore the wavelength of maximum absorbance, the absorption coefficient, the specific refractive increment and the partial specific volume must be calculated with maximum precision and used to ascribe mass values to the proteins in solution.

Purified transthyretin, α1-acid glycoprotein, transferrin produced as described above and a new preparation of α1-antitrypsin were used for calibrating CRM 470. For C-reactive protein the WHO

reference material was used taking 1 IU as equivalent to 1 mg. Two preparations of highly purified kappa and lambda immunoglobulin light chains were produced, one by the reduction of intact immunoglobulin molecules from human serum, the second from a pool of monoclonal light chains (Bence Jones protein) derived from human urine. These were compared with a light chain value obtained by calculation based on molecular mass from the immunoglobulin values ascribed to BCR CRM 470. In the case of α1-antichymotrypsin two preparations of the protein purified from human serum were produced by different techniques. A recombinant protein was synthesised in E.coli from the human gene. The molecular characteristics of the molecules in the purified proteins and in CRM 470 have been established by electrophoretic and immunochemical techniques. All of these materials have been assessed for appropriateness in the value transfer protocol that we have developed and assigned mass values as outlined above.

However, because of the availability of only a few such proteins and the urgent need for a new international reference material, it was decided to calibrate the material for the other proteins against the relevant World Health Organisation (WHO) materials for International Units (IU) and against the best available materials for mass/volume units. For the majority of proteins this was the United States National Reference Preparation for Serum Proteins (USNRP Lot No.12-0575C), from the Centers for Disease Control and Prevention in Atlanta, Georgia, U.S.A.

Value assignment

The analyses for value assignment of albumin, ceruloplasmin, haptoglobin, α_2-macroglobulin, C3, C4, IgG, IgA and IgM were performed by 27 laboratories in Europe, the United States, and Japan. The data from all these laboratories showed agreement and statistical evaluation showed that it was homogeneous and could be merged. For the purified proteins α1-acid glycoprotein (orosomucoid), α1-antitrypsin (α_1-protease inhibitor), transferrin, transthyretin (prealbumin), and C-reactive protein values were assigned in three expert laboratories with good agreement. Multiple dilutions of the CRM 470 and of the relevant primary reference materials were made and linearity over the range of assays and similar regression characteristics were required in order to ensure similar behaviour of the materials (i.e. absence of matrix effect differences) and lack of antigen excess. The ratio of concentrations of the reference and of CRM 470 was used to assign the values. All reconstitutions and dilutions were weight corrected with a sensitive balance.

Values were assigned for α1-antichymotrypsin and kappa and lambda light chains of immunoglobulins in 5 laboratories. In the case of α1-antichymotrypsin the purified preparations showed excellent agreement and the combined values have been used for certification. The recombinant α1-antichymotrypsin was compared with CRM 470 using radial immunodiffusion as only small quantities of the protein were available and would have been insufficient for the volume and concentration requirements of nephelometry and turbidimetry. It was immediately apparent that, in contrast to the proteins purified from human serum, this material did not show a parallel dose response curve to CRM 470. Fig 2 shows clearly this lack of commutability with a steeper curve for the recombinant material implying reduced reactivity with the antibodies used in the measurement. If fewer epitopes are recognised then the rings of precipitation in RID are larger as the antigen is less easily precipitated by the antibody, an effect commonly seen when monoclonal immunoglobulins are compared with their polyclonal counterparts. The recombinant protein contains no carbohydrate side chains which make up 25 % of the native molecule and the main purpose of this work was to establish whether this would prevent an accurate value assignment to the protein in serum and thus whether recombinant proteins were suitable for this kind of work. We conclude that the immunoreactivity of the recombinant protein is considerably lower than that of the carbohydrate-containing serum protein but that the difference between the two materials is reduced with increasing dilution. It is possible that antibodies against epitopes created by the presence of the carbohydrate moiety of the molecule are an important component of the antiserum.

As might be expected the values obtained from the different approaches for the certification of light chains differ markedly. For both K and L pooled monoclonal light chains provided a higher value than light chains derived from polyclonal immunoglobulins. Values in both cases differed significantly from those obtained by calculation (14) and values obtained by radial immunodiffusion differed from those of nephelometry and turbidimetry. All three sets of light chain values are incompatible with the total amount of light chains theoretically present on the basis of the ascribed values for IgG, IgA and IgM. We conclude that purified light chains, whether monoclonal or polyclonal, cannot be used to assign values for light chains bound to immunoglobulins accurately.

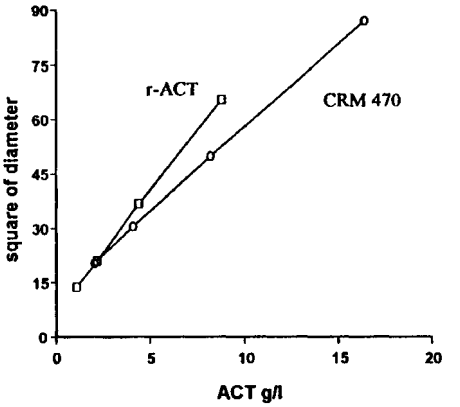

Fig. 2. Dilution curves for RID of r-α1-antichymotrypsin and CRM 470. The value for the lowest dilution of CRM 470 was interpolated on the r-α1-antichymotrypsin curve and the other values calculated from this. The two materials do not show proportional dilution curves (Reproduced from report to BCR).

Availability and Use of the RPPHS

CRM 470 has been released in Europe by the Community Bureau of Reference of the Commission of the European Communities and has been approved by the U.S. Food and Drug Administration for distribution in the United States by CAP. It is available together with the protocols and statistical analysis used for the value assignment upon request from BCR, Commission of the European Communities, Directorate General for Science, Research & Development, DG X11/C/5, Measuring & Testing Programme, Rue Montoyer 75, B-1040 Brussels, Belgium and from the College of American Pathologists, 325 Waukegan Road, Northfield, Illinois 60093-2750, USA.

It is intended that the material be used as a serum-based reference material for transfer of values to tertiary materials (calibrants and controls) and not for direct use in laboratory assays. It is to be hoped that the use of this common calibrant world-wide for serum protein analysis will result in a demonstrable improvement between laboratories and kits. It is the intention of the committee to assign values for further proteins to the CRM 470 as time and funds will allow. Important proteins for future consideration include the immunoglobulin subclasses.

The material is now in use world-wide and quality assurance surveys are already demonstrating an improvement in between-laboratory variance in several countries.

Reference Ranges

Significant changes in reference values have occurred for some proteins, (notably, IgM, α_1-acid-glycoprotein, α1-antitrypsin, transthyretin and transferrin) when compared with values assigned with some reference materials. The generation of new reference ranges based on CRM 470 is thus an important objective for the future. However a number of studies have already been undertaken and several professional societies and diagnostic companies have agreed to use consensus reference ranges in the mean time.

References

1. D.S. Rowe, S.G. Anderson and B. Grab. *Bull Wld Hlt Org.* **42**, 535 (1970).
2. D.S. Rowe, B. Grab and S.G. Anderson. *Bull Wld Hlth Org.* **46**, 67 (1972).
3. C.B. Reimer, S.J Smith, W.H Hannon, R.F Ritchie, L. van Es , W. Becker, H. Markowitz, J. Gauldie and S.G. Anderson. *J Biol Stand.* **6**, 133 (1978).
4. C.B. Reimer, S.J. Smith, T.W. Wells, R.M. Nakamura, P.W. Keitges, R.F. Ritchie, G.W. Williams, D.J. Hanson and D.B. Dorsey. *J Biol Stand.* **9**, 393 (1981).
5. C.B. Reimer, S.J. Smith, T.W. Wells, et al. *Am J Clin Pathol.* 77, (1982).
6. D.W. Tholan and H.A. Homburger. *Pathologist.* **38**, 226 (1984).
7. D.G. Bullock, G. Dumont, A. Vassault, et al. *Clin Chim Acta.* **187**, (1990).
8. S. Baudner, J Bienvenu, S. Blirup-Jensen, A. Carlstrom, A.M. Johnson, A. Milford Ward, R. Ritchie, P.J. Svendsen and J Whicher. *The certification of of a matrix reference material for immunochemical measurement of 14 human serum proteins. CRM 470.* Community Bureau of Reference, Commission of the European Communities, Brussels, pp. 1-172 (1993).
9. A.M Johnson. *Arch Path Lab Med.* **117**, 29 (1993).
10. S. Blirup-Jensen, A. Carlstrom, P.J. Svendsen, S. Baudner, J. Bienvenu, A.M. Johnson, R. Ritchie, A. Milford Ward and J.T. Whicher. *Klinisk Kemi I Norden.* **5**, 9 (1993).
11. S. Baudner. *J Clin Lab Anal.* 7, 278 (1993).
12. J.T. Whicher, R.F. Ritchie, A.M. Johnson, S. Baudner, J. Bienvenu, S. Blirup-Jensen, A. Carlstrom, F. Dati, A.M. Ward and P.J. Svendsen. *Clin Chem.* **40**, 934 (1994).
13. J.T. Whicher and S.W. Evans. *Clin Chem.* **36**, 1269 (1990).
14. M.M. Lievens. *J Clin Chem Clin Biochem.* **27**, 519 (1989).