Quality-control surveys in recent years, in various parts of the world, have shown poor between-laboratory agreement for measurements of plasma proteins. Despite the existence of international reference materials distributed by the World Health Organization, standards produced by diagnostics manufacturers and professional organizations differ significantly in their ascribed values. The reasons for this are complex but include poor availability of the primary materials, confusion about their use, and the fact that their turbidity on reconstitution precludes their use in modern optical immunoassays. This unfortunate situation led to an important initiative to produce sufficient quantities of a widely available, optically clear secondary reference material for plasma proteins that could be used worldwide by manufacturers, professional organizations, and laboratories. Here we present an overview on how the laboratory community, including manufacturers, clinical laboratories, professional societies, and regulators, has reached what we consider is a successful conclusion to a difficult problem.

Indexing Terms: standardization/calibration

Unlike assays of ions and some small molecules for which the chemical and physical characteristics are well understood, measurement of large and complex proteins and peptides depends entirely on the comparison of a test sample with a reference material. Several different primary reference materials for serum proteins have been produced in the last 20 years and issued by the World Health Organization (WHO). A large number of secondary reference materials produced by professional and commercial organizations are in use worldwide, to which values have been assigned by various methods in relation to the primary materials. Unfortunately, for reasons that are unclear, the values for some proteins vary by as much as 100% among different secondary reference materials. These variations in analyte values have been evident in quality-assurance surveys, both in the US and in Western Europe (1, 2). This poor state of affairs is in part attributable to a combination of confusion about the use of the primary materials; widespread use of secondary materials, the values of which have drifted from those of their primary counterparts; poor availability of the primary materials; and the unsuitability of older preparations for use with more modern optical measurement systems. Obviously, the use of a single, internationally agreed upon reference material should substantially reduce the between-laboratory variability, especially if a precise method of value transfer from primary materials to operating reference materials is used.

For these reasons, the International Federation of Clinical Chemistry (IFCC) Committee for Plasma Protein Standardization in 1989 began the process of preparing, characterizing, and calibrating a new international secondary matrix reference preparation for 14 plasma proteins: transthyretin (prealbumin), albumin, α1-acid glycoprotein (orosomucoid), α1-antitrypsin (α1-protease inhibitor), ceruloplasmin, haptoglobin, α2-macroglobulin, transferrin, C3, C4, IgG, IgA, IgM, and C-reactive protein. The material was certified by the European Community Bureau of Reference (BCR) as a Certified Reference Material (CRM 470) in mid-1993 and is to be co-released by the College of American Pathologists (CAP) in mid-1994. CRM 470 is intended for use as a secondary matrix reference material, from which values will be transferred to working calibrants and controls for immunoassay of serum proteins (3).

Previous Reference Materials

In 1967 Rowe et al. (4, 5) prepared a pool of serum to serve as the first International Standard for plasma proteins—the immunoglobulins (4). Part of this pool, batch 67/86, became the WHO Immunoglobulin Standard; filled into ampoules, it was lyophilized at the National Institute for Biological Standards and Control in London. The remainder, processed by the Wellcome Research Laboratories, Beckenham, UK, using a slightly different procedure, subsequently became batches 67/95,
67/97, and 67/99. Batch 67/86 was defined as having 100 units per ampoule for each of the three immunoglobulins and should be thought of as the primary reference material for immunoglobulins with values in International Units (IU). Values for the proteins in 67/95 and 67/97 were derived by direct comparison with the first batch (67/86), by means of radial immunodiffusion. Values for 67/99 were calculated from the mean weight of the ampoule contents of 67/99 and 67/86. These three lots are thus secondary reference materials. Mass values were subsequently ascribed to the primary reference preparation for IgA, IgG, and IgM (67/86) by 10 expert laboratories using immunoechemical measurements against purified proteins. Despite the wide variance among laboratories, mean values were allocated nonetheless (6).

In 1973 the WHO and the International Union of Immunological Societies proposed the production of a new, transparent, stable, lyophilized, pooled human serum to act as a calibrant for several clinically useful serum proteins. Five candidate preparations were prepared, one of which was chosen as a reference material for human serum proteins. One-half of this material was lyophilized in vaccine vials (1.5 mL/vial), sealed with rubber stoppers, and stored at the Centers for Disease Control and Prevention (CDCP; Atlanta, GA) as the US National Reference Preparation (USNRP). The remaining half was lyophilized in glass vials (1.3 mL/vial) and sealed by fusing the glass. The latter became the WHO Reference Preparation for six Human Serum Proteins (WHO 6HSP) (6). International Units were arbitrarily assigned to WHO 6HSP for six proteins (albumin, $\alpha_1$-antitrypsin, ceruloplasmin, $\alpha_2$-macroglobulin, transferrin, and C3c—making it, therefore, the primary reference material for these proteins in IU) and, by transfer with use of single radial immunodiffusion, from WHO 67/86 for the immunoglobulins (for which it is a secondary reference material) (7).

Mass concentration values were subsequently assigned to the USNRP by 24 expert laboratories (7, 8). For the immunoglobulins this resulted in a unit-to-mass conversion factor that differed somewhat from that described by Rowe et al. in 1972 (5) for WHO 67/86, although the difference is surprisingly small considering the improvements in technology in the intervening period. The USNRP is thus de facto the primary reference material for values ascribed in mass units for all the proteins contained therein (the earlier allocation of mass values for immunoglobulins to 67/86 can be considered as superseded by the USNRP as a result of improved techniques).

A new reference preparation produced by CAP, the Reference Preparation for Serum Proteins Lot 1 (RPSP-1), was calibrated at the same time as the USNRP. RPSP-1, a recalcified and delipidated plasma, was used as a reference material by many manufacturers and laboratories until the supply was depleted. CAP subsequently prepared RPSP-2 and RPSP-3; in each case, values were assigned by consensus in comparison with the preceding reference material (9). Over the years, the values drifted somewhat from those originally assigned from the USNRP.

In 1990 the CAP Standards Committee recognized that stocks of RPSP-3 would be depleted months or years before the RPSPHS became available. At the suggestion of manufacturers of immunochemical reagents and instruments, to minimize further changes in value assignments, CAP decided to use the same primary reference materials and the method for value transfer planned for the RPSPHS. Mass concentrations were so assigned; however, the CDCP conversion factors for International Units were used rather than International Units derived from the WHO standards (as in the case of the RPSPHS). RPSP-4 has been available from CAP since early 1991.

New International Reference Preparation for Proteins in Human Serum (RPSPHS)

Since the release of the WHO International Reference Standard for Immunoglobulins (67/86), WHO 6HSP, and USNRP (lot no. 12-6575C) by the CDCP, much has been learned about the requirements for reference materials to be used in modern optical immunoassay systems. After careful evaluation of existing reference materials, investigators felt that certain important criteria must be fulfilled in the preparation of the new RPSPHS.

Of major importance is the requirement that the RPSPHS behave in assay systems similar to the serum samples presented for routine testing. The proteins present within the material should thus, if possible, be in a similar physical state to those in fresh serum and show no alteration on storage. This was achieved by using naturally clotted serum (avoiding the use of thrombin, which may cause protein degradation, and clotting of anticoagulated plasma, which produces inconsistent turbidity with time) and by adding protease inhibitors before lyophilization to ensure preservation during processing. For the most part, the early reference materials for plasma proteins were more turbid than fresh serum, and this turbidity tended to increase with time over prolonged storage because of precipitation of residual fibrinogen incompletely removed by recalcification of plasma. The effect was a decrease in the signal-to-noise ratios and an accompanying decrease in precision in nephelometric and turbidimetric assays. Optimal clarity in the RPSPHS was achieved by collecting blood from volunteers after an overnight fast; allowing spontaneous clotting of the blood in glass; rejecting any donations that were visibly turbid, jaundiced, or hemolyzed; and absorbing the remaining lipoproteins with microparticulate silica. This procedure was extremely effective; however, it caused a significant decrease in overall volume and a reduction in concentration of those analytes with an affinity for the silica particles, such as C4 and IgM.

Allotypic differences in serum proteins resulting from racial and regional variations have become an important issue in the management of analytical data for serum proteins in recent years. Thus, demographic information about the donors to the RPSPHS was recorded.
including α₁-antitrypsin and haptoglobin phenotypes, to allow a similar donor pool to be reassembled in the future for the production of further material. Blood units containing IgM rheumatoid factor and monoclonal components that could interfere with immunochemical assays were excluded. Because of social concerns about the potential hazards presented by serum pooled from large numbers of donors, the individual donations were tested for various infections agents, including those mandated by law, and excluded if found to be reactive. As a result, considerably more attention has been devoted to the constitution of the new reference preparation than to any previous material.

Ideally, primary reference materials for which all values are assigned against purified and highly characterized proteins are desirable. However, given the availability of only a few such proteins and the urgent need for a new international reference material, the Committee decided in 1989 to proceed with development of a preparation calibrated against the relevant WHO materials for International Units (IU) and against the best available materials for mass/volume units. Methods for purifying transferritin, α₁-acid glycoprotein, and transferrin had previously been developed by a Working Group on Plasma Protein Standardization of the IFCC. Proteins prepared by the University of Copenhagen, using these protocols, were used for value transfer to RPHS.

As has been known for years, the original material used for assigning mass values to USNRP for α₁-antitrypsin has been superseded by modern preparations that give very different calibration values. Indeed, the use of these latter preparations has been the major cause of the marked between-calibration variation for this protein. It was thus decided to use a new preparation of α₁-antitrypsin prepared by the Clinical Chemistry Laboratory, Malmo General Hospital, Malmo, Sweden. For C-reactive protein, the WHO reference material was used, i.e., 1 IU = 1 mg USNRP (lot no. 12-0575C), from the CDCP, was used for the assignment of mass/volume units for the remaining proteins.

Preparation of RPHS

Extensive testing of the procedures to be used for the final lot of the RPHS was undertaken with several pilot batches. These procedures were shown to result in a very clear material that could be used in all common methods of immunoassay. Accelerated degradation studies showed no significant change in protein concentration in samples of the lyophilized material stored at 45°C for 1 year. All procedures and data for the final lot were documented in detail to permit the reproduction of similar material when a new lot is needed (10). The steps in preparation were as follows:

1) Fresh serum, derived from naturally clotted whole blood (~175 mL per donor), was collected from several hundred healthy individuals in five European cities. So that a similar donor pool could be assembled in the future, demographic data for each donor were recorded, including country of origin, race, age, gender, weight, and blood group.

2) The individual collections were tested for HIV-1 and -2, HTLV-1, hepatitis B surface antigen, and hepatitis C antibody, with test materials approved by the US Food and Drug Administration, Washington, DC. They were also tested for the presence of rheumatoid factor, monoclonal immunoglobulins, and other abnormalities identifiable by serum electrophoresis. Phenotyping was performed for α₁-antitrypsin and haptoglobin. The individual collections were examined for hemolysis, hyperbilirubinemia, and turbidity. All collections that showed abnormalities or possible interfering substances were excluded.

3) The collections were preserved by the addition of sodium azide, then frozen and shipped to the central processing center. The individual collections were thawed, pooled, and delipidated with fumed microparticulate silicon dioxide, and then stabilized with additional sodium azide, aprotinin, and benzamidine. Pure C-reactive protein was added to a final concentration of ~40 mg/L. The material was buffered to pH 7.2, and subjected to sterile filtration. Vials were filled with 1.00 mL of the material, freeze-dried, and sealed.

Value Assignments

Analyses for value assignment were performed by 27 laboratories in Europe, the US, and Japan, according to a rigorous protocol designed to test the appropriateness of the candidate preparation as well as the performance of the collaborating laboratories (Table 1). Multiple dilutions of the RPHS and of the relevant primary reference materials were made, such that all assays were within the same assay range for both materials. Linearity over the range of assays and regression through zero were required (11) to ensure similar behavior of the materials (i.e., absence of matrix effect differences) and lack of antigen excess. The slopes of the regression lines were used to assign the values. A trial exercise in value assignment clearly showed greatly improved precision if all reconstitutions and dilutions were weight-corrected with a sensitive balance. Therefore, all reconstitutions and manual dilutions used in the value assignment were done in this way.

The values assigned to the RPHS in some cases significantly differ from those in previous reference materials, except for RPSP-4 (currently available from CAP).10 The most significant changes from the mass values assigned to RPSP-2 and RPSP-3 involve transferritin, α₁-acid glycoprotein, α₁-antitrypsin, and IgM. These changes range from 10% to 40%. In addition, the relationships of mass values to IU are different in the RPHS, because the IU values were assigned directly against WHO materials rather than against USNRP.

The evidence from the value assignment exercise sug-

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10 However, referenced against RPHS, the values assigned to RPSP-4 for α₁-antitrypsin and transferritin should be ~10% higher (assigned values, 1.60 and 0.26 g/L, respectively; referenced against RPHS, 1.75 and 0.28 g/L, respectively).
Table 1. International reference preparations used in value assignment of the RPPHS.

<table>
<thead>
<tr>
<th>Protein</th>
<th>USNRP, lot no.</th>
<th>Pure proteins</th>
<th>C-reactive protein, WHO</th>
<th>Complement, WHO lot no.</th>
<th>Immunoglobulins, WHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transferrin</td>
<td>12-0575C</td>
<td>mg/L</td>
<td>85/506</td>
<td>5/4</td>
<td>87/86</td>
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<tr>
<td>Albumin</td>
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<tr>
<td>α₂-Acid glycoprotein</td>
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<td>α₁-Antitrypsin</td>
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<tr>
<td>Ceruloplasmin</td>
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<tr>
<td>α₁-Macroglobulin</td>
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<tr>
<td>Haptoglobin</td>
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<tr>
<td>Transferrin</td>
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<tr>
<td>C3/C3c</td>
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<tr>
<td>C4/C4c</td>
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<tr>
<td>C-reactive protein</td>
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<tr>
<td>IgG</td>
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<tr>
<td>IgA</td>
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<tr>
<td>IgM</td>
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</tbody>
</table>

suggests that the IU values in USNRP (and RPSP-4) are incorrect and should not be used. Although WHO 6HSP and USNRP are part of the same pool, WHO 6HSP was filled with 1.3 mL (to be reconstituted with 1 mL), whereas USNRP was filled with 1.5 mL (to be reconstituted with 1 mL). There should thus be a 14% difference in protein concentration between the two materials. This difference was confirmed in the current value assignment exercise, with a mean difference for the three immunoglobulins of 14%. If, on the other hand, the IU/mL values declared on the package inserts are compared, the average deviation is 8.7%; only in the case of transferrin and the immunoglobulins is it about 14%.

The concentrations of ceruloplasmin, C4, and IgM are low in the RPPHS, relative to those in the fresh normal serum, a result of their strong affinity for the silicon dioxide used in delipidation. This is particularly important for C4 and ceruloplasmin, the concentrations of which are at the lower limits for assay in some instruments (e.g., the Beckman rate nephelometers).

Availability and Use

RPPHS has been released in Europe by the Community Bureau of Reference of the European Economic Community (10). The material has been approved by the US Food and Drug Administration for distribution in the US by CAP.

The IFCC Committee intends that the RPPHS 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. The current lot should last for several years if used in this way. The Committee strongly recommends the use of a value-transfer protocol similar to that used for assignment of values to the RPPHS (10). The important aspects of this protocol include weighing all reconstitutions and dilutions; assaying several dilutions of each material, with dilutions made so as to be in the same assay range for the different materials; assaying replications of samples; and, more important, performing runs on multiple days, each with calibration of the instrument. Because of the experimental design and the simplified statistical analysis, linear regression through the origin was used. The protocols and statistical analysis used for the value assignment of the RPPHS are available 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) or CAP (325 Waukegan Rd., Northfield, IL 60093-2750).

Conversion to the new RPPHS will result in inconvenience and possible confusion to users of protein data. Significant changes in reference values will occur for some proteins (notably, IgM, α₁-acid-glycoprotein, α₁-antitrypsin, transferrin, and ceruloplasmin) if they were assigned with some older reference materials. These changes have already been made in the calibration of CAP RPSP4 (see footnote 10). Reassignment of reference ranges will thus be necessary, either through analysis of new reference groups or by the use of conversion factors supplied by manufacturers of commercial protein calibrants. The Committee is embarking on a project to establish reference ranges, based on this material, for populations in Europe and the US. Valuable work has already been done in this regard by Nordkem (Denmark).

We can hope that the use of a common calibrator worldwide for serum protein analysis will result in a demonstrable improvement in overall performance among laboratories and kits. However, the innate molecular heterogeneity of proteins and the changes that occur in disease will ensure that the problem of accurate protein measurement will never be completely solved (1). It is the intention of the Committee to assign values for additional proteins to the RPPHS as time and funds allow. The BCR has already funded a project to assign
values for $\alpha$-antichymotrypsin and $\kappa$ and $\lambda$ light chains of immunoglobulin. Important proteins for future consideration should include the immunoglobulin subclasses.

We acknowledge the financial support of the BCR and the contribution made by many diagnostic companies, notably Behring Diagnostics, Beckman Instruments, and Dako Corp. We thank the IFCC Scientific Division for their encouragement and support and CAP for their cooperation. We thank the following for their help in this project: Stephen Goodall, The General Infirmary, Leeds, UK; Thomas Leduc, Foundation for Blood Research, Scarborough, ME; Elizabeth Colinet and Christos Proflhis, Brussels, Belgium; Patricia Gembala, CAP; and Robert Nakamura, Scripps Clinic and Research Foundation, La Jolla, CA.

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