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5.2 A New International Reference Preparation for Proteins in Human Serum

S. Blirup-Jensen and P. Just Svendsen

Clinical Immunochemical Department, DAKO A/S,
2600 Glostrup, Copenhagen, Denmark

Introduction

A new international *Reference Preparation for Proteins in Human Serum (RPPHS)* was released jointly by the *European Bureau Communitaire de Réference (BCR)* and the *College of American Pathologists (CAP)* in July 1993. It has been prepared by the Committee on Plasma Proteins Standardization of the *International Federation for Clinical Chemistry (IFCC)* and is approved by the BCR Certification Committee as a *Certified Reference Material (CRM 470).*

CRM 470 is intended for use as a serum matrix reference preparation from which values are to be transferred to working calibrators and controls used in quantitative immunochemical determinations of serum proteins. It should **not** be used directly in routine laboratory assays.

During the past 10 years a large number of reference materials for serum proteins have been used worldwide. These materials have values assigned by a variety of methods against several different primary materials. As a result the values for a given protein may vary as much as 50 - 100% depending upon the reference material used. The variation in analyte values has been obvious in quality control surveys both in the United States and in Western Europe (4, 15). Although the solutions to this problem are complex the use of a single international reference material by all manufacturers and laboratories should reduce the variability to a substantial degree. At the same time a new reference material would be suited to modern technology like turbidimetry and nephelometry and carry assigned values also for analytes previously unaddressed.

In 1989 the IFCC Committee on Plasma Protein Standardization began the process of collecting, preparing, characterizing and calibrating a new international reference preparation for immunochemical measurement of 14 human serum proteins:

Transthyretin*)	α_1-Antitrypsin	Haptoglobin	C3 Complement	IgG
Albumin	Ceruloplasmin	Transferrin	C4 Complement	IgA
α_1-Acid Glycoprotein**)	α_2-Macroglobulin		CRP	IgM

*) Prealbumin
**) Orosomucoid

Since the release of the World Health Organization International Reference Standard for Immunoglobulins (67/86) and for Six Human Proteins and the United States National Reference Preparation (USNRP Lot No. 12-0575C) by the Centers for Disease Control much has been learned about the requirements for reference materials to be used in modern optical immunoassay systems (8, 9, 10, 11, 12).

Ideally, primary reference materials with all values assigned against purified and highly characterized proteins are desirable. However, because of 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 World Health Organization (WHO) materials for International Units (IU) and against the best available materials for mass/volume units. Methods for purifying Transthyretin, α_1-Acid Glycoprotein, and Transferrin together with a method for dry mass determination of proteins in one Electrolyte had previously been developed by a Working Group on Plasma Protein Standardization of the IFCC (2, 3). Using these protocols proteins of the highest purity and with the best physical characteristics available, were prepared by the Protein Laboratory at the University of Copenhagen (DK) for this purpose.

It has been clear for years that the original material which was used for assigning mass values to USNRP for α_1-Antitrypsin has been superseded by modern preparations which give very different calibration values. Indeed the use of such preparations has been the major cause of the marked between-calibrant variation for this protein. It was thus decided to use a new preparation of α_1-Antitrypsin prepared by the Clinical Chemistry Laboratory, Malmö General Hospital, Malmö (S) (7). For C-Reactive Protein the WHO reference material was used taking 1 IU as equivalent to 1 mg. The United States National Reference Preparation for Serum Proteins (USNRP Lot No. 12-0575C), from the Centers for Disease Control Prevention (CDCP) was used for the remaining proteins.

Below is given a summary of the preparation of and the value assignment to RPPHS/CRM 470. A detailed description is given in the BCR-report (1).

Preparation

The preparation of the RPPHS/CRM 470 consisted in brief of:

1) Collection of fresh serum from several hundred healthy individuals in 5 European countries. Demographic data for each donor were recorded including sex, race, age, weight, blood group and country of origin.
2) The individual collections were tested for the following infectious agents: HIV-1 and HIV-2, HTLV-1, hepatitis B surface antigen and hepatitis C virus antibody.
3) Furthermore, the collections were tested for the presence of rheumatoid factors, paraproteins and other abnormalities identifiable by serum electrophoresis. Phenotyping of each donor for α_1-Antitrypsin and Haptoglobin was performed.
4) Examination of the individual collections for haemolysis, hyperbilirubinaemia and turbidity, and exclusion of all collections showing abnormalities or possible interfering substances.
5) The remaining collections were pooled, delipidated and conserved with Sodium Azide, Aprotinin and Benzamidine, and pure C-Reactive Protein was added.
6) After a sterile filtration, vials were filled (1.0 mL/vial), freeze-dried and sealed.

Value assignment to RPPHS/CRM 470

The goal of the value transfer was to assign concentration values (in g/L) to selected proteins in the target material using accepted reference preparations. In order to do this, transfer methods have been selected and transfer protocols have been worked out.

Reference Preparation ⇒ *(Transfer Method + Transfer Protocols)* ⇒ Target Material.

The target material is defined as the serum protein matrix with unknown concentration values (in this case RPPHS/CRM 470). The transfer procedure involved assignment of concentration values to the 14 proteins listed above.

The reference preparation is defined as the protein preparation with known concentration values. In this value assignment, only pure protein preparations or internationally recognized serum protein reference materials were used as mentioned above.

The transfer methods were well established and recognized routine methods such as turbidimetry, nephelometry and single radial immunodiffusion. However, slight variations in the assay principle, in the programming of the instruments or in the reagents may lead to different results. This has lead to the necessity of *method standardization* (14). To minimize these factors precise *transfer protocols* with detailed parameter settings for a number of major instruments were developed and confirmed in a trial value assignment exercise.

A new approach was developed for the value assignment to serum protein preparations (13). By applying strict theoretical considerations and identifying statistically significant and consistent sources of error, two mathematical models have been derived:

1) Value transfer from one serum protein preparation to another serum protein preparation. (Direct value transfer).
2) Value transfer from a pure protein preparation to a serum protein preparation. (indirect value transfer).

Both models are designed as multiple point value transfer, covering the dynamic measuring range for a specific protein. All possible measures are taken to minimize and correct for statistically significant sources of error, e.g. all volumes dispensed are controlled by weighing, and then converted to volume by dividing the weight by the density of the solution. In the first phase, the transfer procedure involves interpolation of the signal obtained for different dilutions of e.g. the reference material R on a calibration curve made from the target preparation T (fig. 1):

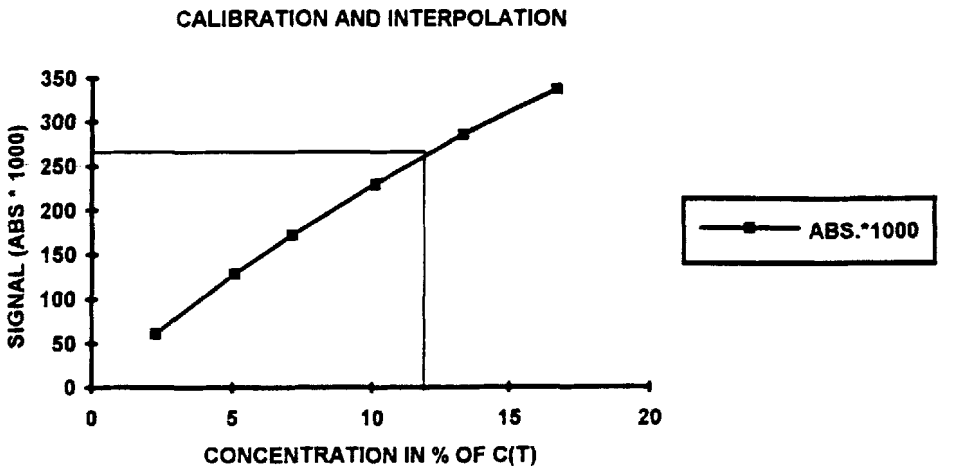

Fig. 1. *When signals of dilutions of R are interpolated on the "calibration" curve (based on T) the concentrations are obtained as relative concentrations, e.g. as percentages of the concentration in T.*

The relative concentrations of R measured in T units are then in the second phase plotted against the weight-corrected dilutions of the reference material (fig. 2)

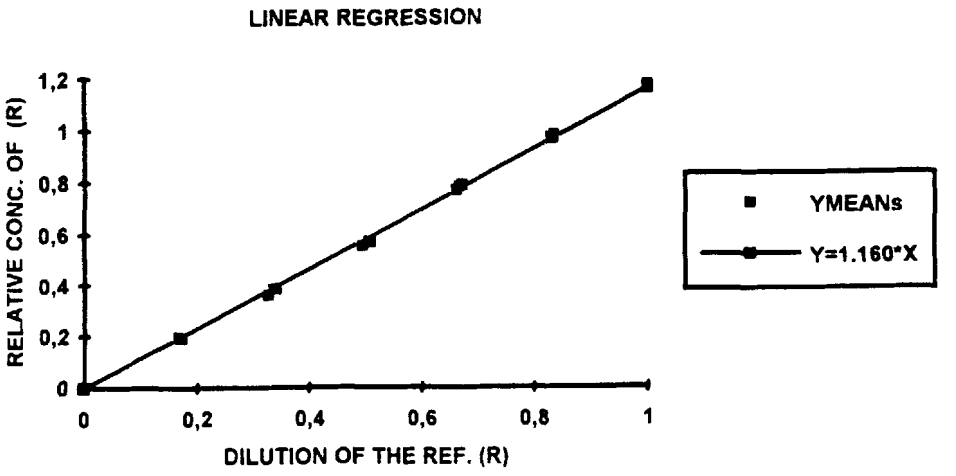

Fig. 2. *The relative concentrations of (R) (in T units) plotted against the mass corrected dilutions of the reference (R). If there is no matrix effects the regression line passes through the origin (0,0) with a slope equal to the concentration ratio C(R)/C(T). As C(R) is known, C(T) can easily be calculated.*

If the two materials behave similarly in the assay (i.e. there is no matrix effects) then the slope of the regression line is equal to the ratio between the concentration of the actual protein in the target material and in the reference preparation.

This transfer procedure was used with great success, and data from 27 different laboratories in Europe, USA, and Japan form the basis of the certified values for the new international reference preparation (PPHS/CRM 470). The standard deviations obtained were remarkably low. This constitutes the best evidence of a very successful value assignment.

Availability and use of RPPHS/CRM 470

The new international reference preparation (RPPHS/CRM 470) is now available as of July 1993 through either BCR or CAP. The material has been approved by the U.S. Food an Drug Administration for distribution in the United States.

The IFCC Committee intends that RPPHS/CRM 470 is to be used as a serum-based matrix reference for the transfer of values to tertiary materials (calibrators and controls) and not for direct use in laboratory assays. The current lot (91/0619) of RPPHS/CRM 470 should last for several years if used in this way. The IFCC committee strongly recommends that the transfer of values from RPPHS/CRM 470 to other reference or control materials are performed using protocols similar to those used for the assignment to RPPHS/CRM 470. The protocols include weighing of volumes used for the reconstitutions and dilutions, assaying of several dilutions of the two materials being involved, with replication of samples and runs, and using appropriate methods of statistical analysis including linear regression through the origin (0,0) (5, 6).

The use of RPPHS/CRM 470 will not eliminate all variations in analyte values, but it will definitely minimize them. However, it is important to stress that the use of authorized transfer methods and - protocols is very essential for obtaining correct value assignments.

It is to be hoped that the use of a common calibrator world-wide for serum protein analysis will result in a demonstrable improvement between laboratories and kits. However, the innate molecular heterogeneity of proteins and the changes which occur in disease will ensure that the problem of accurate protein measurement will never be completely solved. It is the intention of the committee to assign values for further proteins to the RPPHS/CRM 470 as time and funds will allow.

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