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5. Traceability of Concentration Values

5.1 Establishment of a New Reference Preparation for 14 Plasma Proteins/CRM 470 = RPPHS Lot 5

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On January 1, 1990 the committee on Plasma Protein Standardization (PPS) of the International Federation of Clinical Chemistry (IFCC) was established. The members of this Committee are:

J.T. Whicher, Leeds/Great Britain (Chairman)
S. Baudner, Marburg/Germany
J. Bienvenu, Lyon/France
S. Blirup-Jensen, Glostrup/Denmark
R. Ritchie, Scarborough/USA

Further scientists working as associated members did take part in this committee.

Their task was to develop a new reference preparation for human plasma proteins which could be accepted worldwide and which would substitute the established WHO International Reference Preparations which had proved themselves as unsuitable for some modern immunoassays.

5.1.1.1 Quality Requirements to the New Reference Material

After some preliminary discussions it became the definite object of the Committee on PPS to reconcile the European and American tendencies as a basis recommendation for the standardization of plasma protein determination. Such a reference preparation would have to fulfil the following quality aims and claims:

* all serum samples for the pool from healthy blood donors only with complete anamnesis and with established demographic constitution
* absence of infectious diseases (certified)
* serum pool immunochemically suitable for as many plasma proteins as possible

* physico-chemical characteristics of proteins clearly documented
* suitable for the majority of the commercially available immunoassays
  - method dependent bias very low -
* longterm stability/long shelflife (>5 years)
* suitable as an official calibrator
* available in large quantities

5.1.1.2 Milestones

The above mentioned requirements and the studies to obtain the necessary stability data were accepted and initiated by the **B**ureau **C**ommunitaire de **R**eference of the European Communities (**BCR,** Brussels/Belgium) and by the **C**ollege of **A**merican **P**athologists (**CAP,** Northfield/USA), two institutions which have supported the IFCC project by financing various steps.

Blood collection and the necessary characterization of the samples were organized to start in February 1990. The new matrix reference material was produced as a stabilized serum pool in December 1990. The filling and the freeze drying operations were performed in June 1991 after a previous successful trial using a pilot batch lot 0100/36. The final product was labeled with lot 91/0619.

The freeze dried ampoules of the final product were divided into two groups with the following labeling:

a. For the European Users (BCR)
Commission of the European Communities
BCR Reference Material for Proteins in Human Serum
CRM No 470 - CAP/IFCC Lot 91/0619
for in vitro diagnostic use only/for 1 ml

b. For the American Users (CAP)
College of American Pathologists
Reference **P**reparation for **P**roteins in **H**uman **S**erum
CAP/BCR/IFCC Lot 91/0619
for in vitro diagnostic use/for 1 ml

A value assignment was performed for the following 14 plasma proteins:

IgG, IgA, IgM, Albumin (ALB), Transferrin (TF)
all mentioned in the German Quality Control Guidelines (RiliBÄK)

Transthyretin (Prealbumin/TTR), α_1-acid Glycoprotein (A1AG), Haptoglobin (HPT), α_1-Proteinase-Inhibitor (α_1-Antitrypsin/A1AT), α_2-Macroglobulin (A2M), Ceruloplasmin (CER), C3, C4, CRP (by spiking).

A detailed report of the IFCC Committee on PPS on the protein serum standard was released by BCR: the material was named CRM 470, which stands for **C**ertified **R**eference **M**aterial for immunochemical determinations of 14 human serum proteins.

5.1.2 IFCC Material Lot 91/0619 - Blood Collection

Human serum obtained from spontaneously clotted blood of healthy blood donors was used to prepare the reference preparation for human plasma proteins. The human serum samples were provided by five different European blood centres: two actions for blood donation each from Sheffield and Danderyd (Table 1). All together there were seven different blood collections, carried out by:

The Karolinska Institute, Danderyds Hospital, Danderyd/Sweden
The National Blood Transfusion Service, Sheffield/Great Britain
The Centre National de Transfusion Sanguine, Orsay/France
Der Schweizerische Blutspendendienst, Bern/Switzerland
Die Behringwerke AG, Marburg/Germany

Haemolytic and lipaemic sera were discarded after an optical control. The preservatives sodium azide (0.5 g/l) and the proteinase-inhibitor aprotinin (80,000 KIU/l) were added to the selected samples. Thereafter they were frozen by means of liquid nitrogen and stored at - 70°C for further use. Small quantities of the samples of every blood donation were stored separately and used for various analytical tests in accordance with the "Requirements for Biological Substances/Reference Reagents" of the WHO. A medical report was available for each blood donor. Only serum donations which fulfill the characteristics and requirements for "healthy blood donors" were further processed. The exclusion of monoclonal immunoglobulin classes IgG, IgA, IgM was also ensured. Data of concentration for cholesterol, triclycerides, glucose and GOT were obtained: they were all normal. The phenotypes of α_1-Proteinase Inhibitor and Haptoglobin were checked. Blood group, age, sex and race of the blood donors were listed. Serum samples containing factors which could possibly have an influence on the results of an immunoassay like rheuma factors (>30 IU/l), haemolytic components (Haemoglobin >40 mg/l) were discarded. Finally 364 blood donations (donors 20 to 60 years old; 65% male) were found to be suitable and were used for the IFCC reference material.

Table 1

CRM 470 : RPPHS Lot 5 / Collection of Blood

Single Pool Lot No.	Source	Date	Blood Donors	Individual Pool Volume (l)	Individual Pool Protein (g/l)
1. MR 0100/12	Marburg/Germany	February 5 - 8, 1990	55	9.4	70.0
2. BE 0100/18	Berne/Switzerland	February 5 - 12, 1990	44	9.0	74.0
3. SH 0100/39	Sheffield/Great Britain	February 5 - 8, 1990	57	9.5	69.1
4. SH 0100/40	Sheffield/Great Britain	September 3 - 9, 1990	56	10.2	67.6
5. DS 0100/41	Danderyd/Sweden	March 6 - 9, 1990	45	7.1	67.7
6. DS 0100/42	Danderyd/Sweden	September 18 - 21, 1990	53	10.2	71.3
7. OR 0100/38	Orsay/France	February 8 - March 20, 1990	54	9.0	69.6

5.1.3 IFCC Material Lot 91/0619 -Stabilization, Filling Operation and Lyophilization

5.1.3.1 Requirements for Stabilization

As discussed in a series of Committee meetings it was agreed upon that the stabilization procedure had to fulfil especially the following requirements:

* possibly complete elimination of the lipoproteins/lipids
  VLDL, IDL, LDL, HDL and Cholesterol
* no or only minimal blank value
  no or only minimal matrix effects
* no or only minimal denaturation of the protein structure
* no or only minimal reduction of the protein structure for the declared proteins

Treatment by Aerosil® was the option chosen for the stabilization of the reference material. Behringwerke AG had the task of performing this treatment.

Table 2

CRM 470 - RPPHS Lot 5
Lipids/Apolipoproteins before and after Stabilization

Single Pool Lot No.	before Stabilization (g/l) Cholesterol	Triglyceride	Apo A-I	Apo B
1 MR 0100/12	1.85	0.95	1.50	1.15
2 BE 0100/18	1.96	1.16	1.50	1.10
3 SH 0100/39	1.91	0.93	1.43	1.02
4 SH 0100/40	2.02	1.30	1.43	1.15
5 DS 0100/41	2.05	1.09	1.43	1.23
6 DS 0100/42	2.06	0.96	1.50	1.15
7 OR 0100/38	1.81	0.80	1.50	0.95
Single Pool Lot No.	**after Stabilization (g/l) Cholesterol**	**Triglyceride**	**Apo A-I**	**Apo B**
1 MR 0100/12	-	0.0157	-	-
2 BE 0100/18	-	0.0167	-	-
3 SH 0100/39	-	0.0121	-	-
4 SH 0100/40	-	0.0121	-	-
5 DS 0100/41	-	0.0163	-	-
6 DS 0100/42	-	0.0157	-	-
7 OR 0100/38	-	0.0096	-	-

- not detectable

5.1.3.2 Influence of Treatment on the Blank Value

The frozen blood donations were sent to Marburg at the beginning of December 1990 where they were thawed and then pooled in seven different serum pools according to the place and date of the blood donation. The initial volumes of those single pools varied between 7.1 and 10.2 liters. As had been agreed upon the C3 component was transformed into the stable C3c fragment by treatment with Inulin, after which each single serum pool was stabilized by treatment with Aerosil®.

The success of the stabilization procedure was shown by the data regarding the blank values and yields as well as by the content of lipoproteins before and after stabilization (Table 2). The expected results were obtained for all serum pools.

The successful removal of the lipoproteins, which tend to produce turbidity and matrix effects, can be proven on the basis of the drastically reduced blank values (Table 3): reduction to approximately 10% of the original value in the untreated serum pools, measured under undiluted conditions using a Behring Laser Nephelometer / BLN measuring the signals in Volt.

Table 3

CRM 470 ≙ RPPHS Lot 5 / Blank Value and Yield

Single Pool Lot No.	Blank Values initial / final (Volt)	Yield after Stabilization Volume (l)	Protein Conc. (g/l)
1 MR 0100/12	4.55 / 0.64	5.7	63.2
2 BE 0100/18	5.80 / 0.24	6.0	62.2
3 SH 0100/39	5.77 / 0.78	6.5	69.8
4 SH 0100/40	6.32 / 0.79	7.3	65.3
5 DS 0100/41	4.47 / 0.39	5.0	64.6
6 DS 0100/42	9.35 / 0.56	7.3	66.5
7 OR 0100/38	1.82 / 0.62	5.2	69.6

5.1.3.3 Influence of Treatment on the Protein Concentration

It was necessary to concentrate the volume of the single pools after treatment with the stabilizing Aerosil®. An average loss of volume of approximately 32% for the material had to be taken into account in order to obtain the original plasma protein concentration in the final pools. No great difference in concentration before and after stabilization was found for the following eight plasma proteins:

IgG, IgA, Albumin, Transferrin, α_2-Macroglobulin, Transthyretin (Prealbumin), acid α_1-Glycoprotein, Haptoglobin.

A small reduction (< 10%) was observed for
α_1-Proteinase Inhibitor (α_1-Antitrypsin) and C3c.

A clear reduction (on average 30%) was observed for
IgM, C4 and Ceruloplasmin.

Such reductions could not be avoided because the mentioned proteins also have a certain affinity to the stabilizing material, although it is not as strong as that known for lipoproteins.

5.1.3.4 Influence of Treatment on the Protein Structure

The protein structure - representative for the immunochemical reactivity - was also investigated using physicochemical procedures. It had to be proven that there were no structure alterations and no proteolytic digestions during stabilization. This was especially well recognizable on the basis of the completely intact structure for the protein inhibitors α_1-Proteinase Inhibitor and α_2-Macroglobulin. The presence of intact protein inhibitors is very important because they represent a natural protection against any proteases possibly present in such preparations. Possible alterations of the protein structure were also checked for other proteins (section 5.1.5.4).

5.1.3.5 Preservation of the Final Pool

After fulfilling the set quality requirements for each single serum pool, the seven stabilized preparations were mixed into a single final lot (approx. 41 liters) on December 20, 1990. For preservation/proteolytic protection

* sodium azide (to final concentration of 0.5 g/l

* aprotinin = Antagosan of Behringwerke AG (to final concentration of 80,000 KIU/l)

were added to the stabilized lot and this was immediately followed by sterile filtration and storage at - 20 °C/- 30 °C on the same day. The internal denomination IFCC ISRP lot 01 00/34 was given to the final bulk product (without CRP).

5.1.4 IFCC Material Lot 01 00/36 - Pilot Batch

An aliquote of 1.0 liter of the final bulk product obtained on December 20, 1990 and stored at - 20°C/- 30°C, was carefully thawed and used to produce a pilot batch on March 4, 1991. The following substances were added to this small trial batch for further experiments:

* a fraction containing CRP, lot 22 49 0
  (received from Prof. Pepys/Leeds)
  to obtain a final concentration of ca. 40 mg/l CRP
* sodium azide solution (200 g/l)
  to obtain a final concentration of ca. 1 g/l
* benzamidine chloride
  to obtain a final concentration of 0.157 g/l
* HEPES buffer to adjust the pH value to 7.2

The pilot batch was filtrated under sterile conditions and 14 days later, on receipt of the sterility protocol, filled into vials with 1.0 ml which were freeze dried and sealed.

The sterility of the reconstituted lyophilisate was tested and once more confirmed. Further quality requirements regarding homogeneity, dry weight, rest moisture and especially the blank value of the lyophilisate after reconstitution with 1 ml destilled water were fulfilled within an acceptable range. The extremely low blank value, which can be measured in the reconstituted lyophilisate, indicated that freeze drying had no influence on the optical clarity of the material.

Table 4

CRM 470 ^ RPPHS Lot 5 / Preservation / Stabilization

Additives	*Final Concentration*	
Sodium Azide	*max. 15.4 mMol/l*	*(< 1 g/l)*
Benzamidine Chloride	*max. 1.0 mMol/l*	*(< 0.157 g/l)*
Aprotinin	*max 80,000 KIU*/l*	-
HEPES Buffer	*to pH 7.2*	-

** Kallikrein Inhibitor Unit*
1 KIU = 0.0250 APU (= Antiplasmin Unit)
1 KIU = 0.0011 TIU (= Trypsin Inhibitor Unit)

The stability testing program of the pilot batch was performed according to the instructions for stability studies and value transfer given by BCR. No significant alterations in protein concentration (measured by INA and RID) and protein structure (physicochemical analysis) were observed during the period of six months. An acceptable short-term stability of reconstituted material - up to seven days from the time of reconstitution - was also given. Results are identical or similar to those of the final product; therefore details for the pilot batch are reported in connection with the description of the certified preparation = CRM 470 (identical to RPPHS lot 5).

Table 5

Pilot Batch 0100/36 SY and CRM 470 ≙ RPPHS Lot 5 / Final Characteristics

PROPERTIES	PILOT BATCH Lot No. 0100/36 SY	FINAL PRODUCT CRM 470 ≙ RPPHS Lot 5
STERILITY (after filling)	yes, BW/EAB regulation	yes, BW/EAB regulation
	Europäisches Arzneimittel Buch*	
HOMOGENEITY (filling operation)	1.003 mg (n = 5)	1.0024 mg (n = 50) SD = 6.4 mg CV = 0.639 %
DRY WEIGHT OF LYOPHILISATE	73.4 mg (n = 30) SD = 2.02 mg CV = 2.75 %	71.8 mg (n = 30) SD = 2.4 mg CV = 3.34 %
REST MOISTURE	0.59 % (n = 30) SD = 0.14 % CV = 23.7 %	0.57 % H_2O (Fischer) (n = 30) SD = 0.11 % CV = 19.3 %
BLANK VALUE (BLN)	0100/36 SY : 0.40 Volt (n = 30) 0100/36 SL : 0.29 Volt (n = 30)	0100/37 SY : 0.54 Volt (n = 20) 0100/37 SL : 0.46 Volt (n = 20)

S = Serum / Y = lyophil. / L = liquid

* European Pharmacopoea

5.1.5 IFCC Material Lot 91 06 19 - Final Product

5.1.5.1 General Remarks

The final bulk product lot 0100/34 was thawed, spiked with the selected CRP fraction, protected with proportionally increased amounts of the previously reported preservatives (Table 4) and filtrated under sterile conditions. Vials were filled with 1.0 ml, freeze dried under conditions identical to those of the pilot batch and sealed. The date of the filling operation was used to identify the reference material (ISRP lot 91/06 19, i. e. June 19, 1991). The data obtained for characteristics (Table 5) like homogeneity, dry weight and rest moisture of the lyophilisate as well as the blank values before and after lyophilization (reconstituted samples) corresponded very well with those of the pilot batch.

5.1.5.2 Stability Testing by Quantitation before Reconstitution

The stability studies were performed under the same conditions as for the pilot batch lot 0100/36/SY (SY = Serum lyophilized). The studies were performed according to a program proposed by BCR:

Storage Temperatures
- 70 °C/- 20 °C/+ 2 °C to + 8 °C/+ 20 °C (room temperature)/+ 37 °C/+ 45 °C

Time Intervals
months: 1/2/3/6/9/12/18/24 (planned on: 36/48/60)
(for the pilot batch shorter intervals/days: 1/3/7/14/21/28/56/126/192)

Immunochemical Methods
INA = Immunonephelometric Assay (BNA = Behring Nephelometer Analyzer)
RID = Radial Immunodiffusion (Partigen Plates of Behringwerke AG)
After a storage time of 24 months the results indicated vary good stability (Table 6 for INA results). Exception: CRP for samples stored at +37 °C/+45 °C.

5.1.5.3 Stability Testing by Quantitation after Reconstitution

The stability testing of the reconstituted material also indicated an excellent and stable quality of all proteins in the liquid form independent of storage at +2°C to +8°C or at +20 °C. Determinations were made daily up to seven days (short period) and to 28 days (longest period) using a new vial each day. Results were compared to those obtained using a vial stored at - 70 °C. No significant changes were observed for any of the proteins.

Conclusion:

CRM 470 should be used within seven days after reconstitution with 1 ml distilled water when stored at +2 °C to +8 °C after each use. The stability over this period of time is very good, if contamination is avoided.

Table 6

CRM 470 = RPPHS 5

Stability Testing / Method: INA (BNA)
Storage Time: 2 Years
Results in Percent of Material stored at -70°C

	Storage Temperature:					
	-70°C	-20°C	2..8°C	20°C	37°C	45°C
Protein	percent	percent	percent	percent	percent	percent
ALB	100.0%	99.1%	97.5%	98.6%	94.8%	96.0%
A1AG	100.0%	96.3%	93.9%	97.5%	94.5%	93.1%
A1AT	100.0%	95.7%	94.7%	100.0%	100.5%	105.3%
A2M	100.0%	100.6%	100.0%	100.6%	104.7%	104.1%
CER	100.0%	98.0%	101.0%	100.5%	100.0%	102.5%
C3	100.0%	97.7%	98.6%	96.9%	100.1%	100.5%
C4	100.0%	100.0%	100.0%	101.8%	103.0%	106.5%
CRP	100.0%	100.0%	97.8%	95.6%	71.1%	64.4%
HPT	100.0%	93.8%	94.4%	90.7%	95.0%	93.2%
IGG	100.0%	101.0%	99.8%	97.8%	98.6%	97.9%
IGA	100.0%	100.4%	98.4%	100.8%	100.0%	104.9%
IGM	100.0%	100.0%	102.5%	100.8%	105.8%	110.7%
TF	100.0%	98.6%	97.5%	94.7%	95.7%	96.4%
TTR	100.0%	99.3%	101.1%	101.1%	102.6%	104.9%

n = 2

470-030.xls

Reference:
N-Protein Standard Serum, Ch.-B. 067651
N-Protein Standard Plasma PY, Ch.-B. 068034
NA-Latex CRP Kit, Ch.-B. 23613
(Behringwerke AG)

5.1.5.4 Stability Testing by Analysis of Protein Structure

The stability studies regarding the protein structure were performed under the same conditions as those for the quantitation of proteins by RID and INA: i. e. by using samples stored at different temperatures and by testing within the same time intervals. The patterns were compared with those performed immediately after manufacturing. The methods used were:

* **Vertical polyacrylamid gel electrophoresis (PAGE)**
  (P = 8.0%) with and without sodium dodecyl sulfate (SDS)
  to study the structure of various serum proteins.
  No significant alteration was observed.

* **Vertical PAGE** (P = 5.5%) for analysis af A2M
  showed that it mainly existed in the intact (slow) form and no changes to the fast form were observed during the period of testing.

* **Vertical PAGE** followed by **immunoblotting** for CRP:
  The stored samples kept their intact form (exception: +37 °C/+45 °C).

* **Vertical SDS PAGE** followed by **immunoblotting**
  for C3/C3c: The existence of the fragment C3c was confirmed.
  for C4/C4c: The complete form C4 as well as small amounts of fragment C4c were detected.
  for A1AT: The inhibitor-active and intact form corresponded to the majority of the protein present.

* **Two-dimensional immunoelectrophoresis**
  for C3/C3c: All patterns were similar; no further alteration was noted after the initial fragmentation (nearly 100%) to the stable form of C3c and C3d.
  for C4: Only one peak corresponding to the intact form of C4 was observed.
  for A1AT: The majority was present in the intact form together with a small amount of aggregates.
  for CER: Slight degradation of this protein was seen (this is usual after lyophilisation).

Summary of Examination

No distinct alterations in protein structure (Table 7) were found during the period of stability testing up to 24 months referring the samples stored at - 70 °C/- 20 °C/+2 °C to 8 °C/+18 °C to +22 °C.

Table 7

CRM 470 ≙ RPPHS Lot 5
for 14 Plasma Proteins
IFCC/BCR/CAP Material Lot No. 91/0619

PROTEIN STRUCTURES

PROTEIN	STRUCTURE STATE	METHOD FOR DETECTION *
IgG	intact / native	IE; 2-dim.IE
IgA	intact / native	IE; 2-dim.IE
IgM	intact; + aggregates	IE; 2-dim.IE
ALB	intact / native	IE; 2-dim.IE; PAGE/8 % PAA
TRF	intact / native	IE; 2-dim.IE; PAGE/8 % PAA
C3	C3c fragment	IE; 2-dim.IE; PAGE/SDS + IB
C4	C4 slightly fragmented	IE; 2-dim.IE; PAGE/SDS + IB
TTR (PRE)	intact / native	IE; 2-dim.IE; PAGE/8 % PAA
α_1AG	intact / native	IE; 2-dim.IE
α_1PI (α_1A)	intact; + aggregates inhibitor active + types	IE, 2-dim.IE; PAGE/SDS 7 % PAA + IB
α_2M	intact (S-form) inhibitor active	IE; 2-dim.IE; PAGE/5,5 % PAA
CER	slightly altered size unchanged	IE; 2-dim.IE
HPT	intact HP 1-1 ┐ HP 2-1 ├ mixture HP 2-2 ┘ (n = 364)	IE; 2-dim.IE
CRP	intact	IE; 2-dim.IE; PAGE/8 % PAA + IB

* IE = Immunoelectrophoresis
2-dim.IE = two-dimensional Immunoelectrophoresis
PAGE = Vertical Polyacrylamide Gelelectrophoresis
PAA = Polyacrylamide
SDS = Sodium Dodecyl Sulfate
IB = Immunoblotting

Exceptions / Slight Alterations

CER for samples stored at +37 °C and +45 °C after 18/24 months
CRP for samples stored at +37 °C and +45 °C after 18/24 months

Such results of physicochemical analysis confirmed the data of the quantitation by immunoassays.