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5.3 Preparation of the Nordic Protein Calibrator

Ole Blaabjerg[1], Mogens Blom[2], Per Hyltoft Petersen[1], Adam Uldall[3], Hanne Gry[4].

1. Department of Clinical Chemistry, Odense University Hospital, DK-5000 Odense C, Denmark.
2. Department of Clinical Chemistry, Hjørring Sygehus, DK-9800 Hjørring, Denmark.
3. Department of Clinical Chemistry, KAS Herlev, DK-2730 Herlev, Denmark.
4. Medi-lab, Adelgade 5-7, DK-1304 Copenhagen, Denmark.

The problems related to preparation of secondary calibrators for determination of single human serum proteins are numerous. The proteins in the preparation must be genuine and stable for years. Moreover, the preparation must be clear for the use in measurements based on turbidimetric and nephelometric principles. It would, further, be an advantage if the calibrator was prepared from serum from a well defined population so it could be repeatable reproduced.

Commercial available calibrators for specific determination of human proteins are delipidated by treatment by organic solvents or similar materials. This treatment usually denaturates the proteins to a variable extent due to irreversible reactions, and this is often made worse by lyophilizing of the preparation.

Therefore, the aim of the project was to produce a clear and reproducible liquid calibrator with genuine proteins stable for years.

Production of Calibrator

Reproducibility: The reproducibility is obtained by collecting blood from more than 1,000 male blood donors .

The last few mL of blood in the tube after bleeding is collected, left at room temperature for coagulation for one to five hours, centrifuged (2,500*g for 15 min), and the serum aliquots pooled. Solid NaN_3 was added to a concentration of 0.1 % (15 mmol/L), and stored at - 80 °C. The blood donors were all tested for HIV and hepatitis B antibodies, and after a few days any pool where one sample was contaminated was discharged.

Delipidation: The calibrator was cleared by ultracentrifugation, the lipid fraction was removed and the volume restored by 0.9 % NaCl. This procedure is very gentle and keep the proteins genuine without denaturation.

When the sufficient amount of serum is collected, the serum is thawed and recentrifuged (2,500*g for 15 min), carefully mixed, weighed, and then ultracentrifugated for 40 hours at 180,000*g at 4 °C. After ultracentrifugation the lipid layer is sucked off and after a new weighing the calculated amount of 0.9 % NaCl (154 mmol/L with 15 mmol/L NaN_3) is added in order to restore the original weight and the lot is frozen and kept at - 80 °C. When all lots are ultracentrifuged they are thawed and thoroughly mixed, the calibrator is then aliquoted in small portions (2 mL, 5 mL, and 10 mL) in cryotubes, frozen and stored at -80 °C until distribution for determination of the concentrations of the single proteins.

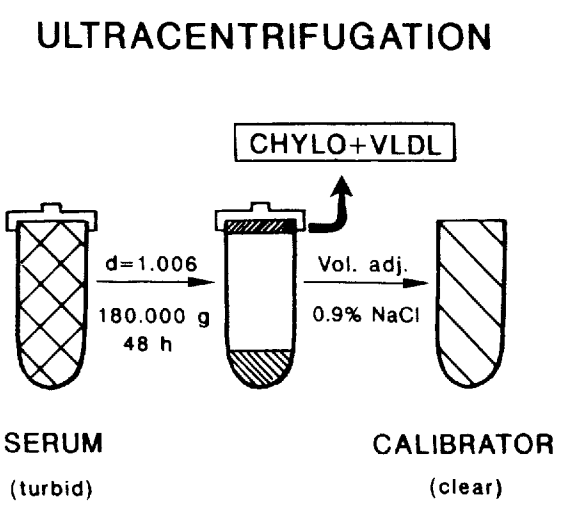

Fig. 5.3.1. Schematic illustration of preparation of calibrator by ultracentrifugation. From Blaabjerg et al. (1) with permission.

Pools: In order to investigate the different steps of the procedure and different storage conditions, several pools were prepared from 1981 to 1989 and kept under different conditions. The pools are

1981: Ultracentrifuged (U-81) kept at - 20 °C (U-81-20) and at - 80 °C (U-81-80) and Not ultracentrifuged (NU-81) at - 20 °C (NU-81-20) and at - 80 °C (NU-81-80).

1983: Ultracentrifuged (U-83) kept at - 80 °C (U-83-80) and
Not ultracentrifuged (NU-83) kept at - 80 °C (NU-83-80).

1984: Ultracentrifuged (U-84) kept at - 80 °C (U-84-80).

1989: Ultracentrifuged (U-89) kept at - 80 °C (U-89-80).

Methods for Documentation

Turbidity of pools: The turbidity of the described batches of pools prepared as the calibrator was measured photometrically.

The extinctions of undiluted pools in 10.0 mm cuvettes were determined spectrophotometrically on a Beckman DU-8 spectrophotometer at 650 nm. The same instrument was used for all measurements.

Genuine proteins: It is difficult to demonstrate that the proteins are genuine. The pools were compared to commercial available calibrators and a fresh serum pool by agarose electrophoresis followed by immunofixation with antibodies from DAKO according to Carlström and Johansson (3).

Table 5.3.1 Measurements of Turbidity in the Pools
Extinction of undiluted pool in 10.0 mm cuvettes at 650 nm

Time of collection of pools	Pool	Time of measurements					
		Nov 81	Nov 82	Mar 83	Apr 84	Oct 84	Mar 89
Sep 1981	NU-81-20	0.587	0.759	0.791	1.111	1.948	2.417
	NU-81-80	0.587	0.768	0.738	0.688	0.915	1.024
	U-81-20	0.060	0.063	0.061	0.095	0.162	0.554
	U-81-80	0.060	0.068	0.063	0.069	0.073	0.079
Jan 1983	NU-83-80			0.365		0.600	
	U-83-80			0.049		0.062	
Mar 1984	U-84-80					0.060	0.062
Au-De 1989	U-89-80						0.044

Combined reproducibility and stability: The stability of the preparations during storage as well as the reproducibility of preparing the pools are measured at the same time by the described procedures. This means that the results reflect both aspects. The measurements were performed at two occasions, in 1984 and in 1989.

1984: Radial immunodiffusion was performed according to Mancini (5).
Electro immunoassay was performed according to Laurell (4).
Turbidimetry was performed according to Blom and Hjørne (2) on a Gemsaec centrifugal analyzer.
Nephelometry was performed on a Technicon AIP Nephelometer®

1989: Measurements were performed on a COBAS FARA centrifugal analyser from ROCHE according to DAKO's recommendations.

For all measurements antibodies from DAKO were used.

Results

Turbidity of pools: The turbidity of the pools was determined at several occasions and the extensions are shown in Table 5.3.1. It is seen from the table that ultracentrifugation is necessary for obtaining stable negligible values. Further, the storage at - 80 °C keeps the extinction low with insignificant increases for up to eight years. It should be mentioned that NU-83-80 was stored at - 20 °C from April 1984, and that the freezer where NU-81-20 and U-81-20 were kept was defect in July 1984 with temperatures up to - 10 °C for one week, but these facts do not change the general tendencies.

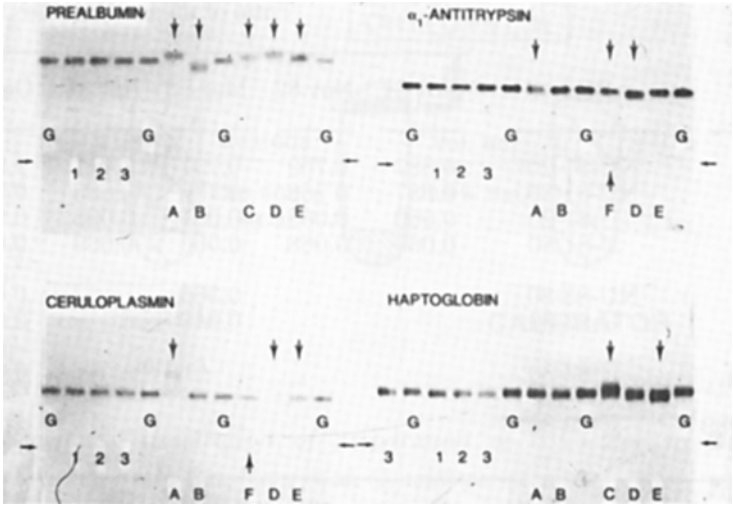

Fig. 5.3.2. *Comparison of three calibrator preparations (1:1981, 2:1984, and 3:1987) with six commercial protein calibrators (A,B,C,D,E, and F) and a fresh serum pool (G) by agarose electrophoresis and immunofixation. Arrows indicate changes in electrophoretic mobility compared to the fresh pool. From Blaabjerg et al. (1) with permission.*

Genuine proteins: Changes in protein mobility can be disclosed by agarose electrophoresis and immunofixation If the mobility of the proteins is changed, this demonstrates changes in electrical charge and is an indication of denaturation. Three calibrator pools (from 1981, 1984, and 1987) and six commercial protein calibrators

were compared with a fresh serum pool by electrophoresis and immunofixation in 1987 and the results from the four proteins, which are most sensitive to denaturation are shown in Fig. 5.3.2.

Prealbumin shows changes in electrophoretic mobility in all the commercial calibrators (indicated by arrows) whereas the three preparations (from 1981 to 1987) of the frozen liquid calibrator all show the same mobility as in the fresh serum pool.

In three of the commercial calibrators α_1-Antitrypsin shows lower concentration, an extra fraction and a broader band, respectively. No changes in mobility or concentration is seen in any of the frozen liquid calibrators.

Ceruloplasmin may be the best indicator of denaturation and four of the commercial preparations show changed mobility and decreased concentrations. Again the frozen liquid pools behave like the fresh serum pool.

Table 5.3.2 Combined Stability of Proteins and Reproducibility of Pools
The data are normalized according to protein and method

	Rad. Imm. Diff			Elec. Imm. Ass			Turbidimetry			Nephelometry		
Protein	81	83	84	81	83	84	81	83	84	81	83	84
Prealbumin	0.978	1.007	1.015	1.001	0.985	1.013	1.011	0.999	0.991	-	-	-
Albumin	0.999	0.992	1.010	1.063	0.966	0.970	-	-	-	1.000	0.998	1.002
Orosomucoid	0.989	0.995	1.016	1.015	0.980	1.005	1.028	0.987	0.985	-	-	-
α_1-Antitrypsin	1.021	1.000	0.980	1.045	0.974	0.981	1.016	0.985	0.999	-	-	-
Haptoglobin	0.993	0.997	1.009	1.017	0.978	1.005	1.012	0.983	1.005	-	-	-
Ceruloplasmin	-	-	-	1.013	0.991	0.995	-	-	-	-	-	-
α_2-Macroglobulin	-	-	-	1.010	0.982	1.008	-	-	-	-	-	-
Transferrin	0.992	1.007	1.001	0.978	1.000	1.022	1.003	1.005	0.993	-	-	-
IgA	1.012	0.991	0.998	0.989	0.997	1.014	1.020	0.984	0.995	-	-	-
IgG	0.998	1.006	0.996	1.020	0.996	0.985	1.015	0.993	0.992	0.992	0.996	1.012
IgM	1.007	0.978	1.015	1.016	0.91	0.989	1.044	0.972	0.984	-	-	-

Changes in Haptoglobin may be more difficult to interpret as the combinations of phenotypes may be different from calibrator to calibrator.

Combined reproducibility and stability: Three preparations of calibrator (from 1981, 1983, and 1884) were compared by quantification of eleven proteins by four methods in 1984. The results for the normalized values are shown in table 5.3.2 according to protein and method.

The results are all close to 1.000 and scattered with a variation close to the analytical. Only for α_1-Antitrypsin (and perhaps IgM) the results are consistent for the three methods indicating about 2 % higher values in the 1981 preparation. This, however, does not indicate instability as the highest values are in the oldest preparation.

A new investigation in 1989 including a new preparation (collected in 1987), however, indicated lower values for the acute phase reactants. In table 5.3.3 the largest differences found between the 1984 and 1987 preparations are shown.

Table 5.3.3 Combined Stability of Proteins and Reproducibility of Pools
The data are given as differences in percentage for 1987 minus 1984

Protein	Mean difference	95 % Confidence Interval
Prealbumin	- 0.91 %	- 2.24 to + 0.42 %
Albumin	- 0.89 %	- 1.89 to + 0.11 %
Transferrin	+ 0.57 %	+ 0.12 to + 1.02 %
IgG	- 0.45 %	- 1.05 to + 0.15 %
IgA	+ 1.80 %	+ 1.60 to + 2.00 %
IgM	- 0.52 %	- 2.32 to + 1.28 %
Orosomucoid	- 4.8 %	- 5.2 to - 4.4 %
α_1-Antitrypsin	- 3.5 %	- 4.1 to - 2.9 %
Haptoglobin	- 2.1 %	- 2.6 to - 1.6 %

The results from table 5.3.3 clearly show lower values for the acute phase reactants in the 1987 preparation. This is not a question of instability as the oldest preparation has the highest values. However, it illustrates that minor fluctuations in mean values of the population occurs e.g due to infectious diseases, whereby, the biological uncertainty is more important than the statistical uncertainty, due to sample size.

Discussion

It has been discussed for a long time whether protein calibrators should be frozen liquid or lyophilized, and how the delipidation should be performed. The IFCC-group decided on chemical delipidation and freeze drying. The documentation given here,

however, demonstrates that ultracentrifugation and storage as frozen liquid pool is very gentle to the proteins (compared to older commercial calibrators) and that the proteins are stable for several years. For α_1-Antitrypsin, however, the quantifications indicate some minor changes in the structure, as described in the following two sections and in chapter 6. Except from this problem (which is well known for other protein calibrators) the frozen liquid calibrator is for years a reliable calibrator with genuine proteins when stored at - 80 °C.

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