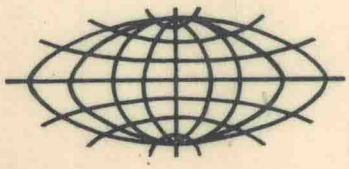
DEVELOPMENTS IN

BIOLOGICAL STANDARDIZATION

«TEST METHODS FOR THE QUALITY CONTROL OF PLASMA PROTEINS»



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PROCEEDING OF THE

« TEST METHODS FOR THE QUALITY CONTROL OF PLASMA PROTEINS »

Proceedings of a Symposium
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INTRODUCTION

Quality control and test methods form the very essence of standardization as far as biologics are concerned. It is, therefore, only natural that IABS organized this symposium for plasma proteins. The field of blood derivatives is not new to our association; it has been dealt with on several occasions and was referred to in a number of presentations at the present conference. New research into the biological activity of substances contained in certain products as trace-impurities only, now permits their exclusion, which in turn is hoped to reduce adverse reactions. Recent regulations on a national or international level result in well defined preparations for which, however, the implications for clinical use are not well understood. Compilation of new methods led to the recognition of new problems, so that the symposium was regarded by the participants not as one similar to many others, but as the beginning of a new series of conventions where practical implications are discussed.

IABS will try to fulfill this demand at annual intervals.

Bern, September 1979

W. Hennessen
President

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International Symposium on Test Methods for the Quality Control of Plasma Proteins, 1979, *Develop. biol. Standard., vol.* **44**, p. 1 (S. Karger, Basel 1979)

SESSION I

PURITY + INNOCUITY

Chairman: *D.L. Thomas (U.K.)*
Rapporteur: *H. Geiger (F. R. G.)*

International Symposium on Test Methods for the Quality Control of Plasma Proteins, 1979, *Develop. biol. Standard.*, *vol.* **44**, pp. 3-10 (S. Karger, Basel 1979)

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GEL FILTRATION IN SEPHADEX G200 FOR THE CONTROL OF PREPARATIONS OF HUMAN IMMUNOGLOBULIN

L. Vallet

ABSTRACT

Gel filtration in Sephadex G200 has been used in the quality control of normal and specific immunoglobulins at the Blood Products Laboratory, Elstree, for more than eleven years. Eluting from a 140 cm column, polymerised and aggregated protein is excluded by the gel, followed in order of elution by fractions containing the IgG dimer and the monomer. After these, smaller protein impurities such as albumin are eluted and then fragments from the proteolytic breakdown of IgG. On a single chromatogram, possibly five distinct peaks may be recorded and their components quantified.

Gel filtration in Sephadex G200 provides an alternative to sedimentary boundary ultracentrifugation, by which fragmentation of IgG can be more readily observed. In addition, the fraction containing the polydisperse aggregates excluded by the gel provides an index of adverse treatment of immunoglobulin during preparation and storage.

From the time of its introduction in 1962, gel filtration in Sephadex G200 quickly found its place in the study of human immunoglobulin, that is the fraction, mainly IgG, which is prepared for clinical use. For the analysis of these preparations, in which polymerisation, aggregation and fragmentation of the IgG molecule may readily occur, this chromatographic medium had unique advantages. James, Henney & Stanworth in 1964 described its use in demonstrating the proteolytic degradation of IgG, and again Stanworth & Henney used it in 1967 in the study of the 10 S or dimeric form of the molecule. Since 1967, gel filtration in Sephadex G200 has been used at the Blood Products Laboratory in the control of immunoglobulin preparations, first for the ether precipitated fraction of Kekwick & Mackay (1954) and later for that of the Kistler & Nitschmann (1962) method. When methods of control are under review, it is perhaps appropriate to give a brief account of experience in the application of gel filtration routinely to control quality rather than more flexibly as a research tool.

APPARATUS & METHOD

Column:	Length 135 to 140 cm. diameter 3.0 cm. Water jacket at ambient temperature 15 to 25°.	
Gel:	Sephadex G200.	
Eluant:	Sodium chloride 0.15M sodium phosphate buffer pH 7.0 7/2 0.15 sodium azide 1/5000 w/v.	2 vols. 1 vol.
Flow rate:	24 ml/hr.	
Sample:	150 mg protein in 3 to 5 ml.	
Analyser:	LKB Uvicord II, fitted with 4 mm rectangular cell.	
Recorder:	Smith Servoscribe 1s with log. scale converter and integrator.	
Chart speed:	30 mm/hr.	

While the apparatus and conditions for analysis by downward flow through the column are given above, some details require additional comment. There have been only minor changes since our first study of immunoglobulin by this method (Kekwick, Mackay, Maycock and Vallet, 1971). In the analyser, the cylindrical cell normally supplied has been replaced with a rectangular cell in order to increase the path length and to improve the linearity of absorbance as a measure of protein concentration (up to 75 per cent of the recorder scale). Using the logarithmic converter, absorbance was recorded directly on the chart together with the integrator trace. A graphically recording integrator was preferable to the digital type because it provided a simple means of obtaining areas once the boundaries between fractions had been located and for correction of the baseline. With later types of analyser, absorbance can be recorded directly without the use of the converter. To prepare the chromatograms which appear in figures 1 to 4, data have been taken from the recorder charts and replotted on a reduced scale against eluted volume so that the various profiles can be readily compared.

From early trials it was found that it was an advantage to use a column of greater length than is conventional. Typical samples of immunoglobulin may be resolved into as many as five characteristic fractions which are denoted by letters A to E in their order of elution. Using a one metre column, the IgG dimer (B) separated from the monomer (C) insufficiently for the areas of their peaks to be defined (figure 1). By increasing the column length to 1.4m the areas could be adequately resolved for measurement. After first extrapolating the trailing curve of peak C to the baseline, this section was reflected about a vertical line through the apex of C to complete the leading side of the peak. With the aid of this construction the positions of boundaries drawn vertically between fractions B and C and between C and D were estimated and the area representing monomer was obtained. Boundaries between minor peaks were located from positions of minima on the curves, aided by reference to chromatograms of typical preparations. Using a well packed column, the monomer peak was symmetrical. If skewness occurred in this peak, the column was repacked. Due to the extended length of the column, each run required at least forty hours.

RESULTS AND DISCUSSION

Fragmentation

With a lower limit of purity by electrophoresis of ninety per cent for gamma globulin, considerable amounts of other plasma proteins may be present as impurities, particularly albumin. The incubation of immoglobulin at 37° for one month, as required in the European Pharmacopoeia II (1971) monograph, provides an accelerated test for fragmentation of IgG by proteases such as plasmin. In the chromatograms for typical immoglobulin batches, albumin and other proteins of this order of size, which appear in fraction D, separate clearly from the IgG fragments which are in fraction E. Results for fresh and incubated samples of a batch which displayed instability on incubation are shown in figure 2. Fraction E increased from 0 to 5 per cent of the total absorbance attribuable to protein. Figure 3 shows extreme results from aged samples, after 17 years in solution at 4°C and in

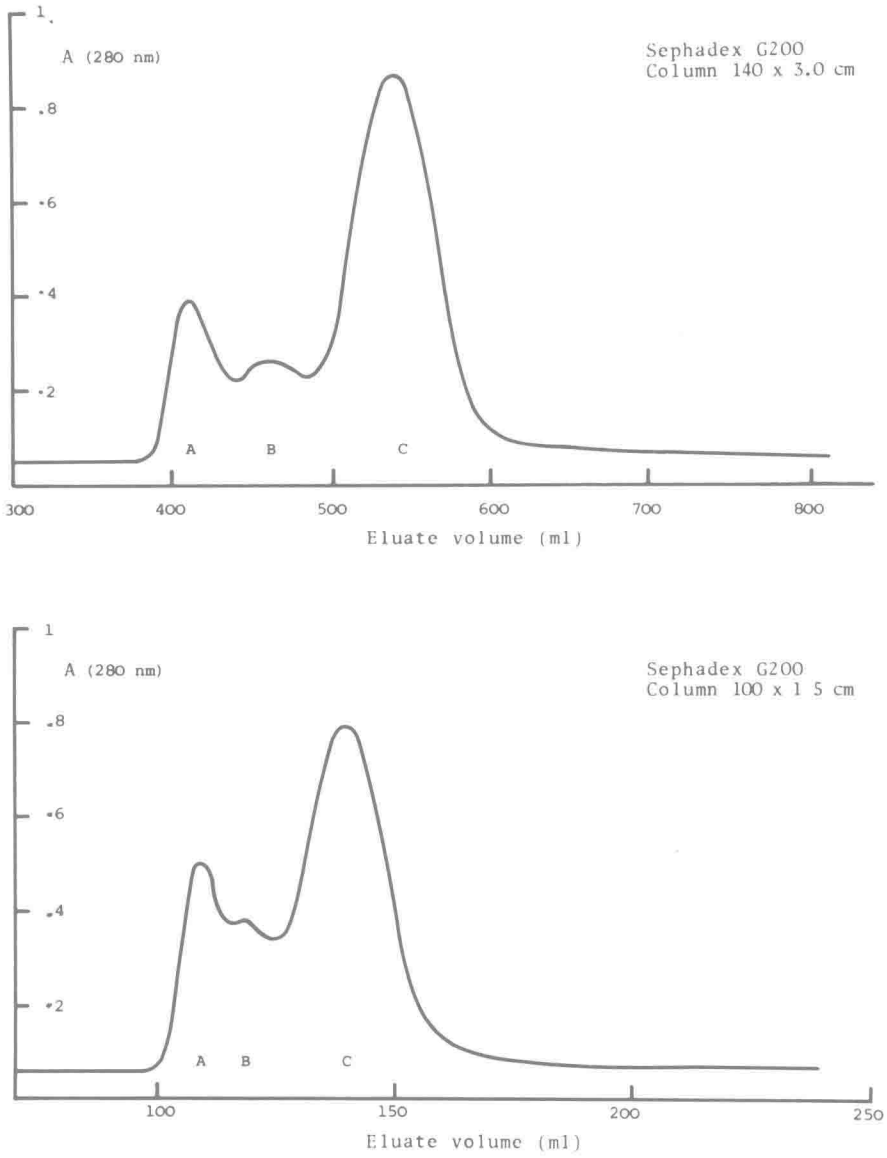

Fig. 1. Immunoglobulin, batch no. GG12.
Gel filtration in Sephadex G200 in 140 cm and 100 cm columns.

the dried state in an ampoule at ambient temperature. The former shows an advanced state of fragmentation, in which an additional small fraction appeared. Breakdown of this magnitude may occur after much shorter periods and it has

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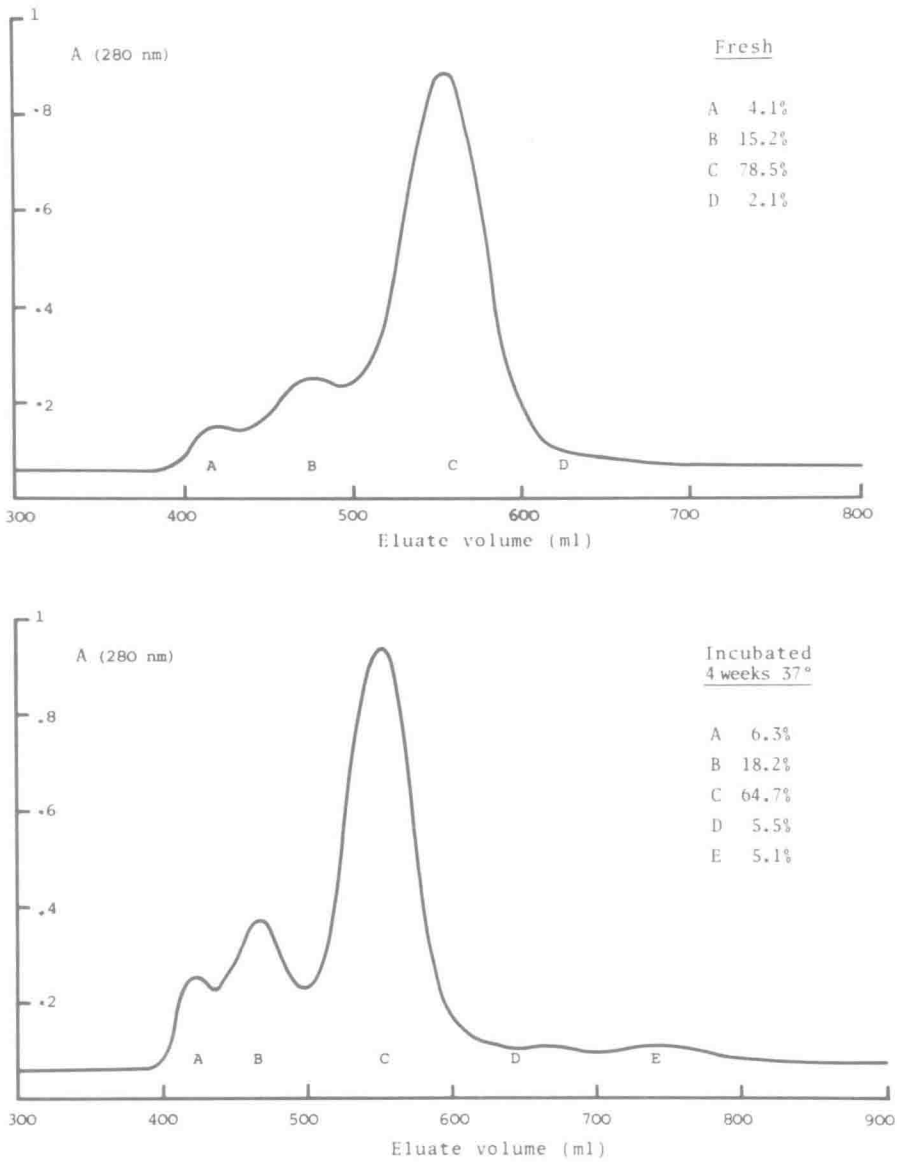

Fig. 2. Immunoglobulin, batch no. GG56.
Gel filtration in Sephadex G200, 140 cm column, of fresh solution and after 4 weeks incubation at 37°.

been seen in a purchased batch of immoglobulin within its expiry date. The aged freeze-dried sample has a chromatogram comparable with those of relatively fresh solutions.

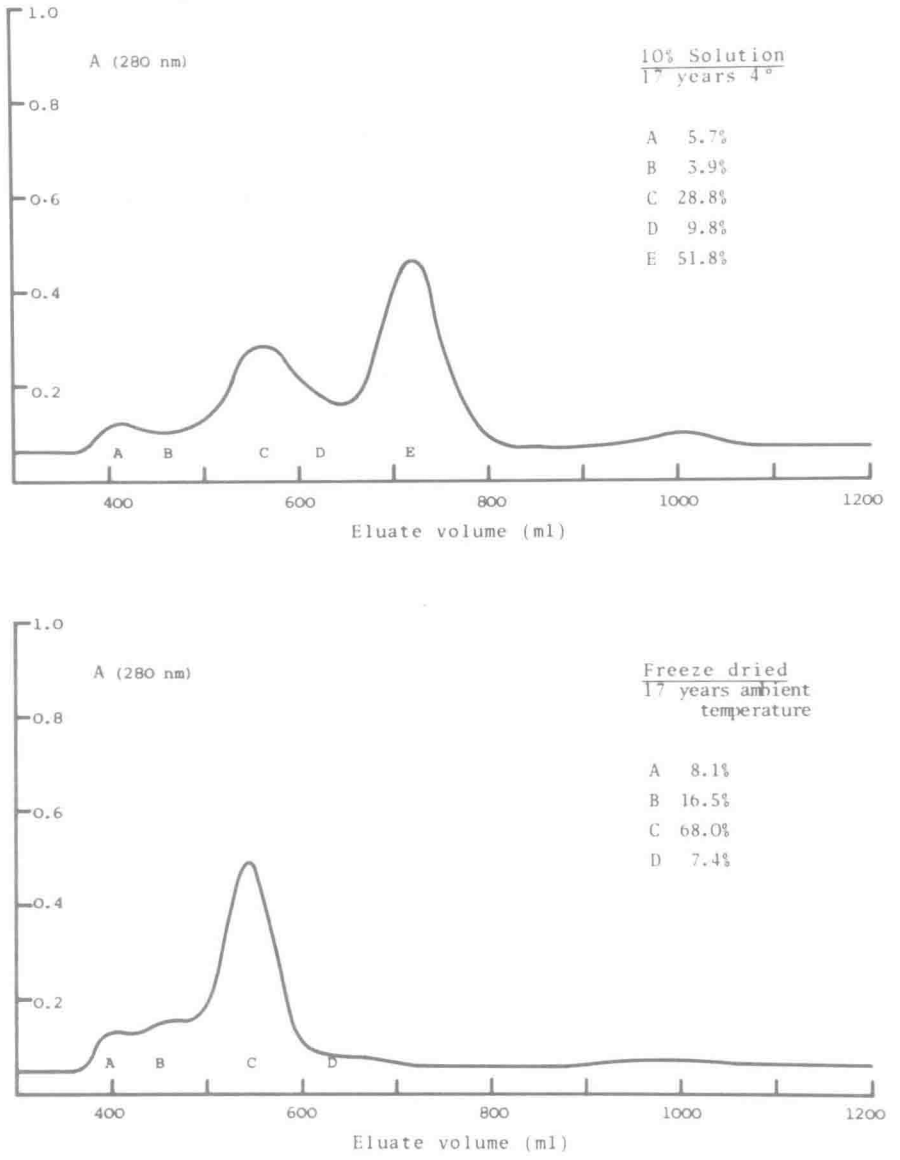

Fig. 3. Immunoglobulin, batch no. LKG11.
Gel filtration in Sephadex G200, 140 cm column, of immunoglobulin solution, 10 % w/v, after 17 years at 4°C; and freeze dried immunoglobulin reconstitued after 17 years at ambient temperature.

Aggregation and Polymerisation

Dimerisation is a property of IgG which has been shown to occur reversibly in solution (Finlayson, Armstrong and Young, 1971). As well as dimers, most

preparations contain material of larger molecular size. In sedimenting boundary ultracentrifugation, this appears to be polydisperse and is not readily observed, though traces of size about 19 S may be identifiable. IgM and alpha$_2$ macroglobulin, though demonstrably present in immoglobulin preparations are unlikely to account for more than a small fraction of the high molecular weight material. Using density gradient ultracentrifugation, Makula, Burnet, Tayot and Plan (1974) found up to ten per cent of the total protein of immoglobulin preparations in a high molecular weight fraction greater than 10 S. Gel filtration in Sephadex G200, by exclusion from the gel, effectively collects the high molecular weight material into one fraction, A, and amounts of this approaching ten per cent were commonly observed. The variation in the amount in this fraction between batches is considerable. A batch containing 8 per cent excluded fraction in Sephadex G200, when run through Sepharose 6B (figure 4) which has a higher exclusion limit showed no clearly defined fraction ahead of the dimer and a reduced amount is excluded. In this range, no molecular size predominates.

As a consequence of this difference between gel filtration and sedimentary boundary ultracentrifugation, fraction B cannot be compared directly with the 10 S component. First the percentages of the chromatographic fractions need to be recalculated, omitting A and adjusting the value for fraction D to allow for the lower extinction coefficient of albumin than immoglobulin at 280 nm. Mackay, Vallet and Combridge in 1973 described stability studies by these and other methods. Data are not presented here because the available analyses were not sufficiently matched in time and conditions of treatment to be strictly comparable for B and 10 S. With regard to effects on equilibrium between the IgG monomer and dimer, Stanworth and Henney observed in 1967 that in gel filtration the fraction B zone migrates in free buffer while in the ultracentrifuge the 10 S sedimentation boundary is between the complete protein solution, less macrocomponents, and the monomer and smaller components. With this fundamental difference in conditions, it is of interest that most values for fraction B and for the 10 S component by sedimentation were within the range twelve to 22 per cent of the total protein observed.

In Table I, results of chromatography of batches prepared as 15 per cent $^{w}/_{v}$ protein solutions in 0.15M sodium chloride, 0.01 %$^{w}/_{v}$ thiomersal from the Kistler & Nitschmann (1962) fraction are summarised. The rather wide range of values for fraction A is evident and experience with earlier batches has shown that it is largely dependent on conditions of preparation of the final solution and its storage. Some increase occurs during incubation at 37°. For specific immunoglobulins which have been prepared as more dilute solutions, this effect is more marked.

At present in the accepted specifications for immunoglobulin, the percentage of dimer, as measured by ultracentrifugation, is subject to control though present knowledge suggests that it is in equilibrium with the monomer, as an intrinsic property of this protein. Limitation of high molecular weight protein possibly because it is not of defined size, other than 19 S, is not subject to such control, though it is potentially more objectionable and is present as an artefact. By the use of gel filtration in a medium such as Sephadex G200, a simple means is available to place limits both on polymerised or aggregated and on fragmented protein in immunoglobulin preparations.

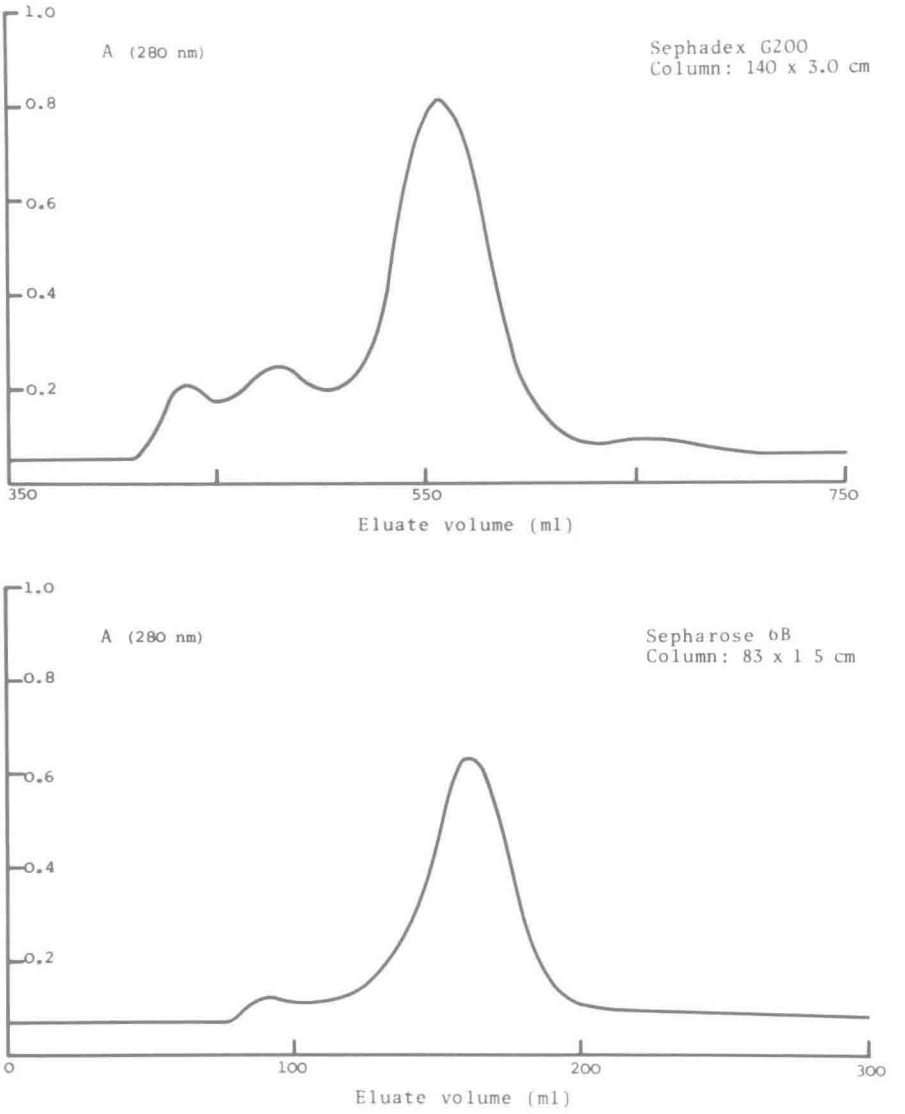

Fig. 4. Immunoglobulin, batch no. GG126.
Gel filtration in Sephadex G200, 140 cm column; and in Sepharose 6B, in 83 cm column.

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Table I. Gel filtration in Sephadex G200 of normal immunoglobulin, as currently prepared by the method of Kistler and Nitschmann (1962).

n = 8	Fraction (% at 280 nm)				
Fresh (stored 4°)	A	B	C	D	E
Mean	4.1	15.6	76.9	3.3	0.1
s.d.	1.3	1.9	2.8	0.7	0.3
Range	2.6 6.7	13.0 18.0	72.4 80.3	2.2 4.6	0 0.8
Incubated (4 wks 37°)					
Mean	5.4	17.9	72.9	3.4	0.4
s.d.	1.7	2.9	4.1	0.6	0.6
Range	3.2 8.5	14.3 21.9	65.0 77.1	2.1 4.0	0 1.4

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DETERMINATION OF THE CONCENTRATION OF PLASMA PROTEINS BY DENSITY MEASUREMENT

A. Gardi and P. Degen

ABSTRACT

Density measurement with a commercially available instrument (DMA 46, PAAR) was studied as an alternative method to the time-consuming and sometimes expensive methods currently in use for the determination of protein concentrations. The method is very useful for protein estimation in salt — and alcohol — free albumin bulk solutions and as an additional end product test for albumin and immunoglobulin solutions; it may also be used in the determination of total protein in plasma.

INTRODUCTION

Since the beginning of industrial plasma fractionation, there has been a need for a rapid and precise method to determine the concentration of protein solutions, especially of albumin and immunoglobulin bulk solutions which have to be diluted to a specified final concentration. The methods currently in use are often expensive and time-consuming, e.g. the Kjeldahl and the Biuret metod.

On the other hand, certain methods require a predilution of the sample, as is the case with UV-absorption measurement.

Recently, several apparatus' for electronic density measurement became commercially available. We therefore studied the suitability of density measurement for the determination of protein concentration in plasma, in albumin bulk solutions and in final solutions of albumin and immunoglobulin. The method is, of course, not proteinspecific but since the composition of the albumin or immunoglobulin bulk solution, resulting from the same fractionation scheme, does not show great variation, this study seemed worth-while.

According to our fractionation scheme (1, 2), the albumin precipitate (Cohn fraction V) is dissolved in water and then passed through a gel filtration column, where alcohol and salts are separated from the protein. If a concentrated albumin solution is to be prepared, the eluate is concentrated by ultrafiltration. Our bulk albumin solutions are therefore alcohol — and essentially salt — free. The sodium content of the diluted bulk solution is as low as 9 milliequivalents (meq)/L; after concentration to about 25 % albumin, it reaches 45 - 50 meq/L. Since the composition of our bulk albumin solution is very constant, this is also the case for the final product after addition of sodium chloride and stabilizers.

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