Journal of Chromatography, 240 (1982) 1–8
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,662

GEL CENTRIFUGATION CHROMATOGRAPHY FOR MACROMOLECULAR SEPARATIONS

KLAUS B. ANDERSEN*,* and MAURICE H. VAUGHAN
Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260 (U.S.A.)
(Received December 16th, 1981)

SUMMARY

A method for chromatographic separation of macromolecules by the use of centrifugation through gel filtration materials is described. The liquid excluded from the gel material is removed by centrifugation; thus, this method offers rapid separation and minimal dilution of samples. Because of the rapid separations obtained, this method is particularly suitable for study of enzyme reactions, ligand binding and formation of intermolecular complexes in general. Within the limitations of the short columns which were employed in the centrifuge, the resolution of macromolecules is equivalent on columns eluted conventionally and columns eluted with the assistance of centrifugation.

INTRODUCTION

Centrifugation methods for desalting macromolecules by gel filtration were first developed for liter volumes, by the use of basket centrifuges[1,2], and later adapted for milliliter volumes[3] and microliter volumes[4], using small centrifugation tubes for support of the gel material. The centrifugation methods have been used both for batch elution[3] and stepwise chromatography[4].

The gel centrifugation technique removes and collects the liquid excluded from the gel by centrifugal force. Its advantages are that separations can be (i) done practically without dilution, (ii) obtained in the order of minutes and (iii) performed on viscous samples.

In the past centrifugation has been applied to gel filtration principally to accelerate simple class separations such as the desalting of macromolecules, on highly cross-linked filtration materials such as Sephadex G-25 (Pharmacia) or Bio-Gel P-2 or P-4 (Bio-Rad Labs.)[5,6]. We have found that it is possible to use less cross-linked materials by which separations of macromolecules can be obtained. The separations can be performed (i) as batchwise separations of large macromolecules from smaller macromolecules or (ii) as stepwise chromatographic analysis of macromolecules. We

* Present address: The Fibiger-Laboratory, Nordre Frihavnsgade 70, DK-2000 Copenhagen Ø, Denmark.

0021-9673/82/0000–0000/$02.75

describe both theoretical and practical aspects of the technique in this communication.

MATERIALS AND METHODS

The gel centrifugation column assembly and the principle of gel centrifugation are illustrated in Fig. 1. The gel materials were supported in 1-, 3- or 10-ml plastic syringes (Becton-Dickinson) without pistons. Porous polyethylene discs (Bel-Art Products) were placed in the bottom of the syringes; the syringes were then filled with slurries of the gel material (Sephadex, Sephacryl and Sepharose series; Pharmacia) and placed in a swinging bucket centrifuge (IEC PR6 centrifuge rotor with a radius of 20 cm), hanging by the syringe shoulder in a centrifuge tube. The gel materials from Pharmacia which were utilized are currently supplied as beads in a single size range, except for Sephadex G-25 and G-50. The fine grade of the last two materials was used, with the exception of a test of G-25 beads of different sizes described in Results.

Operation of the columns was performed as illustrated in Fig. 1. (1) The excluded liquid was centrifuged out of the column, (2) sample was applied and (3) the excluded liquid was centrifuged out and collected. Further chromatographic elution of the column was done by repeated application of buffer and recentrifugation of the column.

RESULTS

Gel centrifugation chromatography is related in principle to conventional "wet" gel filtration chromatography, with the difference that in the former the excluded liquid is fully or partly removed from the gel material. Thus in gel centrifu-

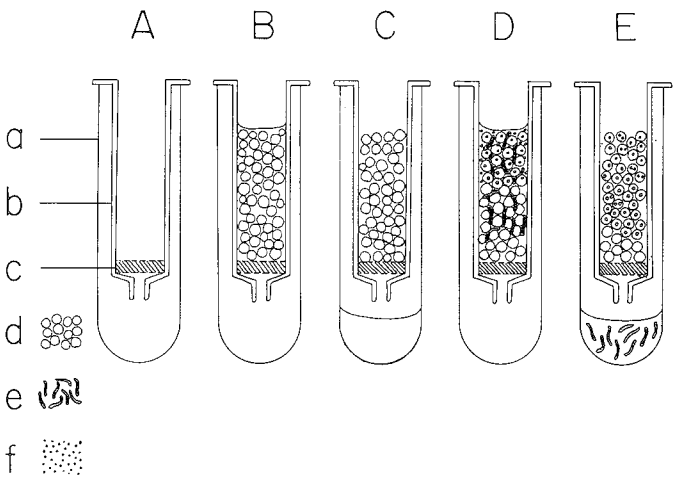

Fig. 1. Centrifugation column assembly and principle of the method. a = Centrifuge tube; b = plastic syringe; C = porous polyethylene disc; d = gel beads; e = large molecules; f = small molecules; the buffer is indicated by the meniscus. A = Assembly; B–E = operation: B, gel slurry applied; C, liquid excluded volume centrifuged out; D, sample applied containing large and small molecules; E, excluded liquid containing the large molecules centrifuged out of the column.

gation chromatography the separations of larger from smaller molecules occur as they pass over the gel beads in a thin layer of excluded liquid.

For a theoretical analysis of gel centrifugation chromatography the gel bed of volume V_t can be divided into the stationary volume included in the gel beads, V_s (gel material volume plus included liquid volume), and the volume excluded from the gel beads, V_0:

$$V_t = V_s + V_0$$

For spherical gel beads the excluded volume is approximately $0.3 \times V_t$ (ref. 7); thus, $V_s = 0.7 \times V_t$. The liquid in the excluded volume is partly removed from the bed by centrifugation

$$V_0 = V_a + V_b$$

where V_a is the volume of the excluded liquid centrifuged out of V_0, and V_b the volume of the excluded liquid remaining in V_0.

A chromatographed molecule is characterized by its elution volume, V_e[7,11], and its partition coefficient K[7,11]. K is a measure of the distribution of macromolecules between the excluded liquid volume and the total included volume; thus, in gel centrifugation chromatography:

$$K = (V_e - V_b)/V_s$$

The elution of a macromolecule in gel centrifugation chromatography can be empirically characterized by the retardation value, R_c, defined as:

$$R_c = V_e/V_t$$

The retardation value for markers excluded from the gel beads ($K = 0$) is therefore a measure of the fraction of the total volume which is contained in the remaining excluded liquid volume, V_b/V_t. Thus, a retardation value equal to zero for such a marker corresponds to removal of all the excluded liquid ("dry gel"), while a retardation value of 0.3 indicates a lack of removal of the excluded liquid ("wet gel").

The force necessary for removal of the excluded liquid from the different gel materials and the maximal force they can withstand is shown in Fig. 2. The maximal force the gel materials are able to resist without collapsing depends on their rigidity. A guideline for the rigidity of the products is given by the manufacturer as the maximal hydrostatic pressure the gel materials are able to sustain[11]. Above the maximal force the gel materials collapse and are pressed out of the centrifugation columns. Sephadex G-10, G-15, G-25, Sephacryl S-200 and Sepharose 6 B can all withstand forces higher than 6000 *g* as indicated in Fig. 2. Materials with increasing molecular weight exclusion limits in the Sephadex and Sepharose series can withstand less force. Thus, Sephadex G-200 can withstand only 120 *g* and Sepharose 2B only 1200 *g*. It must be noted, however, that the maximal force the materials are able to withstand depends on the height of the column, since the stress on the lower part of the column is the total centrifugal pressure of the overlaying material.

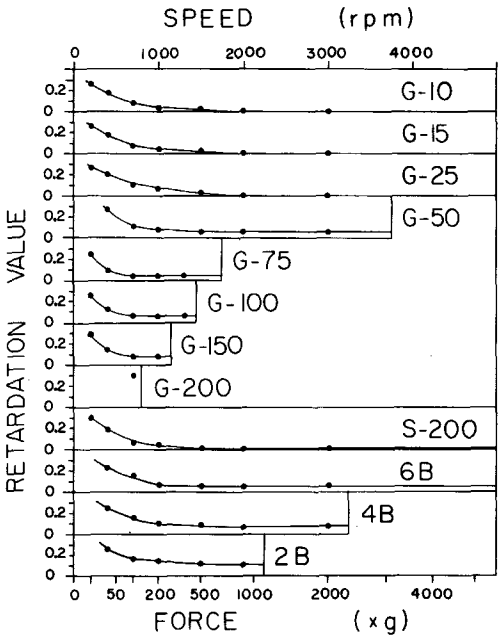

Fig. 2. Properties of gel filtration materials for column centrifugation; the Sephadex, Sephacryl and Sepharose series are indicated with their trade numbers, G, S and B respectively. 10-ml gel beds (55 × 15 mm) were used, in 10-ml syringes. The centrifugation speed above which the gel materials are pressed out of the columns is indicated by the length of the bars. The curves inside the bars indicate the retardation value for Blue Dextran 2000 (for Sephadex and Sephacryl) and bovine milk colloid (for Sepharose), used as excluded markers. The retardation value was calculated from the elution volume, (see text) which was measured by stepwise elution. A 200-μl volume of marker solution was applied and centrifuged for 5 min at the indicated speed, followed by elution steps with 0.5 ml buffer and centrifugation for 5 min at the indicated speed.

At forces less than those causing the gels to collapse, the proportion of the excluded liquid removed by centrifugation is a function of the applied force. In Fig. 2 this is indicated by curves which specify the volume of buffer which must be applied to and step-eluted from the column at any particular centrifugal force level in order to result in the quantitative elution of an excluded marker. For example, using Sephadex G-10, at 40 *g*, 0.2 bed volumes must be applied to the column and removed by centrifugation in order to elute Blue Dextran marker.

Removal of 50% of the excluded liquid (retardation value of 0.15 for excluded markers) requires a force between 40 and 100 *g* for 5 min for the different materials. Sephadex G-200, however, did not release its excluded liquid even when centrifuged at 100 *g* for 5 min.

Nearly total removal of the excluded liquid is obtained with Sephadex G-10, G-15, G-25 and Sephacryl S-200, which show retardation values for excluded markers less than 0.01 at forces above 100 *g*. Eighty percent removal of the excluded liquid (retardation value of 0.06 for excluded markers) is obtained at forces above 200 *g* for Sepharose 6B. Above 200 *g*, 75% removal of excluded liquid was obtained for Sepharose 4B and 60% for Sepharose 2B.

The small amount of excluded liquid phase which remains on these dextran

and agarose beads, including those most tightly cross-linked, at even relatively high centrifugal forces is critical to the separations achieved in gel centrifugation chromatography, as discussed below. We have determined the thickness of this liquid layer on three different sizes of Sephadex G-25 beads, coarse, fine and superfine, whose average diameters in the swollen state we estimated by microscopy to be 190, 75 and 25 μm respectively. To determine the size of the bound, excluded layer we equilibrated columns of 7.8-ml bed volume of each bead type with a solution of Blue Dextran, centrifuged them at 900 *g* for 10 min and then eluted the columns twice with 3.0-ml portions of buffer and measured the absorbance at 280 nm of the eluates. The average thickness of the excluded layer was then calculated from the volume of this liquid retained on the column, the diameter of the beads and the volume of the column occupied by the beads, 70%. The results indicated thicknesses of 0.51, 0.28 and 0.26 μm for the layer on coarse, fine and superfine beads respectively. Thus, this layer is quite thin, and its thickness is relatively independent of the diameter of the Sephadex

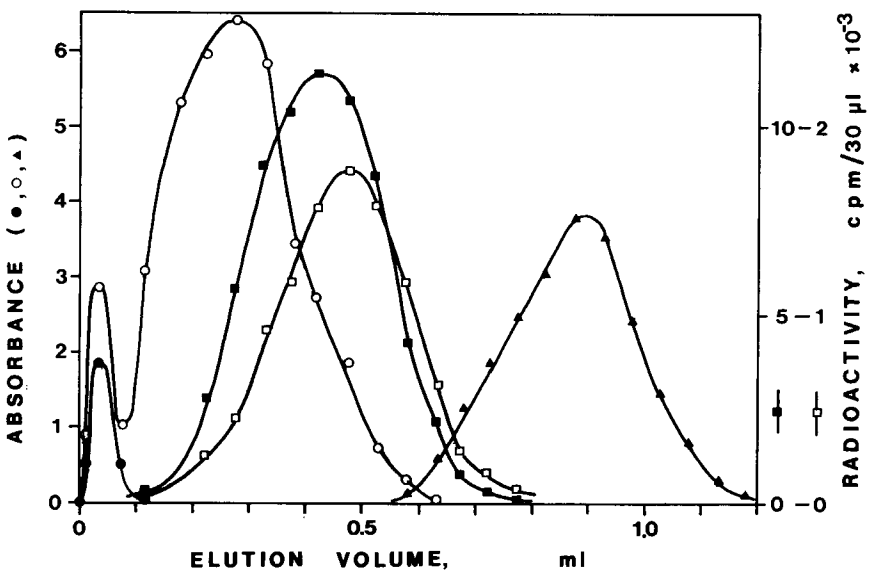

Fig. 3. Stepwise chromatographic separation of a mixture of Blue Dextran, ferritin, albumin, ovalbumin and phenol red on a Sepharose 6B centrifugation column. The column was equilibrated with elution buffer (100 m*M* NaCl, 20 m*M* Tris, pH 7.5) and centrifuged for 3 min for 2000 rpm prior to usage. The column measured 50 mm × 18 mm^2 after centrifugation. An 18-μl volume of a mixture consisting of 120 μg Blue Dextran 2000 (Pharmacia) (●), 250 μg ferritin (Miles). (○), 0.5 μg [^{125}I]bovine serum albumin (120,000 cpm) (Sigma, St. Louis, MO, U.S.A.) (■), 0.2 μg [^{125}I]ovalbumin (19,000 cpm) (Sigma) (□) and 22 μg phenol red (▲) was loaded carefully on the top of the column and centrifuged for 3 min at 2000 rpm. The Blue Dextran was chromatographed prior to the separation on a Sepharose 4B column to exclude molecules with a molecular mass smaller than 2,000,000. The iodinations were performed by the chloramin T method[8]. The column was eluted stepwise with addition of elution buffer and centrifugation for 3 min at 2000 rpm. The first two elution volumes, 16 and 25 μl, were used to wash the mixture carefully into the gel. Thereafter, 50-μl volumes were used. The optical absorption at 630, 430 and 350 nm of the eluates was measured by a Carl Zeiss M4QII spectrophotometer, and the absorptions of Blue Dextran at 630 nm, phenol red at 430 nm and ferritin at 350 nm were calculated from their respective spectra. The amount of ^{125}I-iodinated proteins in the eluates was measured after separation by SDS polyacrylamide gel electrophoresis[9] using a Searle Series 1175 gamma counter.

G-25 beads. This measurement is easily carried out and, as will be discussed, the value of V_b obtained is useful in arranging for optimal separations on any particular column to be used for gel centrifugation chromatography.

An example of the use of centrifugation columns is shown in Fig. 3. This figure illustrates a stepwise chromatographic analysis on a 50 mm × 18 mm^2 Sepharose 6B centrifugation column of a mixture of Blue Dextran (molecular mass larger than 2,000,000), ferritin (molecular mass 470,000), bovine serum albumin (molecular mass 69,000), ovalbumin (molecular mass 46,000) and phenol red (molecular mass 345). Blue Dextran was collected in the excluded volume with an retardation value of 0.04. Phenol red chromatographed at a retardation value of 0.96 and was used as an indication of the total volume of the included plus excluded liquid. As can be seen from the figure, a practically complete separation of albumin and ovalbumin from both Blue Dextran and phenol red was obtained. Ferritin separated totally from the phenol red and well from Blue Dextran. However, ferritin showed a peak in the excluded volume. After analyzing the eluates by SDS-polyacrylamide gel electrophoresis, it was observed that this material mainly consisted of ferritin dimers.

We have compared this separation to a similar separation obtained on an identical column, which was operated by the conventional "wet" technique (not shown). The partition coefficient obtained with the two methods differed less than 0.05 for any of the chromatographed molecules. Furthermore, the resolution obtained with the centrifugation column was similar to that with the "wet" column.

It should be noted that, except for Blue Dextran, the band widths for the compounds were almost identical (0.45–0.50 ml). This similarity of the band widths is normal for gel filtration[10]. The HETP (height equivalent to a theoretical plate)[7] was 0.9 mm for phenol red.

DISCUSSION

The gel centrifugation chromatography method permits a faster separation of macromolecules than conventional "wet" gel filtration chromatography without loss of resolution. A mixture of macromolecules can thus be separated in a few minutes. Enzyme reactions can be stopped and analyzed by gel centrifugation, and binding reactions between macromolecules as well as ligand binding can easily be studied.

The optimal use of the materials in gel centrifugation chromatography is obtained at forces where a maximal volume of the excluded liquid is removed, and the gel bed is not collapsed. These criteria provide a guideline for the optimal use of the materials, as observed in Fig. 2. With the gel materials investigated, separations can be obtained of molecular masses ranging from 500 to 30,000,000 (ref. 11).

Similar gel materials from other sources can also be utilized. As an example we have found that Bio-Gel P-60 (Bio-Rad Labs.) has an optimal usage range in centrifugation chromatography from 100 to 1000 *g*, with a maximal excluded liquid removal of 60%.

The effects of centrifugation on the different gel materials we have utilized vary. With the more tightly cross-linked gels, such as Sephadex G-10, G-15 and G-25, there is very little bed shrinkage on centrifugation, and the excluded liquid which is removed, V_a, is replaced by air. With the other types of gels there is generally some shrinkage on centrifugation at high speed and little or no air appears to enter the

column. Since air has not replaced that portion of the excluded liquid volume removed from these gel types by centrifugation, V_a, there must be a compression of these gel beads. Presumably the individual gel beads lose their spherical shape and pack more tightly, in a manner similar to cells in a tissue. It is also possible that part of the shrinkage of the column might be due to compression of the individual gel beds. This will decrease the pore sizes and thus the exclusion limit. However, although Sepharose 6B columns show some shrinkage on centrifugation, the elution positions of the proteins in Fig. 3 show no significant difference from the positions of the same proteins run by conventional "wet" gel filtration, with respect to the partition coefficients. Thus a compression of the individual gel beads is not likely for this material at least.

The retardation value (0.96) of phenol red in Fig. 3 reflects the tighter packing of the Sepharose 6B beads and smaller air filled volume, V_a. At an excluded volume of $0.04 \times V_t$ the volume of the included liquid becomes $0.92 \times V_t$. V_s which also includes the volume of the gel material is thus slightly larger than $0.92 \times V_t$. As mentioned, V_s is $0.7 \times V_t$ for spherical beads.

A unique practical consideration in gel centrifugation chromatography is the determination of the portion of the excluded volume which is not removed from the column by centrifugation, V_b. This parameter must be taken into account in determining the volume of the sample to be applied to the column and the size of the subsequent elution volumes. These choices will in turn depend upon whether the purpose is to achieve a maximal resolution of a set of different molecular species or a simple class separation, as in desalting a macromolecule.

The role of V_b is best seen by considering the situation under which chromatographic resolution occurs in the gel centrifugation technique. The flow-rate of the moving, excluded liquid phase during centrifugation is very high in comparison to conventional "wet" gel chromatography, and it might thus be expected that only poor resolution would be achieved using centrifugation. However, it has been pointed out[7] that, although high resolution of molecules by "wet" gel filtration requires flow-rates allowing equilibrium partitioning between the mobile, excluded phase and the stationary phase, the limiting diffusion step is not within the gel beads. Instead, the limiting diffusional steps appear to occur *outside* the boundary of the restricted space within the gel particle[7]. We have shown that the average thickness of the liquid layer in V_b on Sephadex G-25 is not more than 0.5 μm or approximately 2% of the radius of the smallest type of G-25 bead. Owing to the small dimensions of this layer, equilibration of molecules partitioning between it and the included, stationary phase should be much more rapid than is the case in "wet" filtration, where the average dimensions of the liquid-filled spaces between beads are relatively large.

Thus, the thinness of the excluded layer in V_b explains the relatively high resolution obtained with gel centrifugation chromatography. For this reason, if high resolution is desired, the volume applied per chromatographic step in this technique should not greatly exceed the value of V_b for a particular column, so as to maintain the thinness of that layer. It should be noted that the discontinuous, step-wise operation of the column in this method is not actually disadvantageous, as it permits equilibration of molecules between the included and excluded phases between elution steps. As in "wet" gel chromatography, careful application of samples and elution buffer, in an even band across the column bed top, is essential if optimal resolution is

to be obtained. Operation of chromatographic columns in normal centrifuge tubes in the present study limited column lengths to a maximum of 65 mm. Within this limitation we have obtained similar degrees of resolution between macromolecules chromatographed on gel filtration columns operated in a conventional wet manner and columns eluted with the aid of centrifugation. While such short columns do not permit full exploitation of the resolving capacity of the filtration media, the rapidity with which they can be operated in the centrifuge should make this technique useful for applications where the macromolecules to be resolved constitute relatively simple mixtures of species differing considerably in molecular weight.

The method of centrifugation chromatography has been examined here with respect to gel filtration materials, but ion exchange and affinity chromatography can also be adapted to obtain many of the advantages of gel centrifugation. The chromatographic materials used in these techniques are often very rigid, and only a little force is necessary for removal of the unbound sample and elution buffer. For example, carboxymethylcellulose and DEAE-cellulose (CM 52 and DE 52 from Whatman) require less than 40 *g* for liquid removal (results not shown).

ACKNOWLEDGEMENTS

We thank Ms. Celine Lee for excellent technical assistance, and Pharmacia for supplying information. Klaus B. Andersen was the recipient of a travel grant from the Danish Natural Science Research Council (No. 511-5714). The work was supported by N.I.H. grant CA-10922.

REFERENCES

1 B. Gelotte and A. Emneus, *Chem.-Int.-Techn.*, 38 (1966) 445.
2 N. I. A. Emnéus, *J. Chromatogr.*, 32 (1968) 243.
3 M. W. Neal and J. R. Florini, *Anal. Biochem.*, 55 (1973) 328.
4 M. Krieger and J. Tobler, *Anal. Biochem.*, 81 (1977) 450.
5 L. J. DeFilipp and D. E. Hultquist, *Arch. Biochem. Biophys.*, 1970 (1975) 670.
6 R. C. Kelly and P. H. von Hippel, *J. Biol. Chem.*, 251 (1976) 7229.
7 P. Flodin, *Dextran Gels and their Applications in Gel Filtration*, Pharmacia, Uppsala, 1968.
8 W. M. Hunter, in D. M. Weir (Editor), *Handbook of Experimental Immunology*, Blackwell Scientific Publications, Oxford, 1967, p. 608.
9 U. K. Laemmli, *Nature (London)*, 277 (1970) 680.
10 K. J. Bombaugh, in J. J. Kirkland (Editor), *Modern Practice of Liquid Chromatography*, Wiley-Interscience, New York, 1971, p. 237.
11 *Gel Filtration, Theory and Practice*, Pharmacia, Uppsala.