

GE Healthcare

Amersham Gel Filtration Calibration Kits

Product Booklet

Codes: 17-0441-01 High Molecular Weight
17-0442-01 Low Molecular Weight



Page Finder

1. Legal	3
2. Handling	4
2.1. Safety warnings and precautions	4
2.2. Storage	4
2.3. Expiry	4
3. Components	5
3.1. Components	5
3.2. Other materials required	5
4. Description	6
5. Critical parameters	7
6. Protocol	8
6.1. Preparation of calibration kit	8
6.2. Gel and calibration kit selection	9
6.3. Gel preparation	11
6.4. Column selection	12
6.5. Bed packing	13
6.6. Selection and preparation of calibration standards	15
6.7. Sample application	17
6.8. Measurement of elution volume (V_e)	18
6.9. Preparation of calibration curve	18
6.10. Molecular weight distribution	20
7. Typical results	22
8. Additional information	25
8.1. Important notes	25
9. Related products	27

1. Legal

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2. Handling

2.1. Safety warnings and precautions

Warning: For research use only.

Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes, wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.

2.2. Storage

Store at 2–8°C

It is strongly recommended that solutions of the Calibration Kit proteins and Blue Dextran 2000 be made fresh just prior to their use as calibration standards. The quality of the Calibration Kit proteins when stored in solution is highly dependent on the particular protein and the composition of the buffer.

2.3. Expiry

For expiry details see outer packaging.

3. Components

3.1. Components

Each kit contains 50 mg of each protein and 50 mg of Blue Dextran 2000.

* These proteins are supplied mixed with sucrose or mannitol to maintain stability and aid their solubilization.

High Molecular Weight Gel Filtration Kit 17-0441-01

Aldolase*

Molecular weight (M_r) 158 000, Stokes Radius (Å) 48.1, rabbit muscle.

Catalase*

M_r 232 000, Å 52.2, bovine liver.

Ferritin*

M_r 440 000, Å 61.0, horse spleen.

Thyroglobulin

M_r 669 000, Å 85.0, bovine thyroid.

Blue Dextran 2000

Low Molecular Weight Gel Filtration Kit 17-0442-01

Ribonuclease A

M_r 13 700, Å 16.4, bovine pancreas.

Chymotrypsinogen A

M_r 25 000, Å 20.9, bovine pancreas.

Ovalbumin

M_r 43 000, Å 30.5, hen egg.

Albumin

M_r 67 000, Å 35.5, bovine serum.

Blue Dextran 2000

3.2. Other materials and equipment required

- Gel suitable for the expected M_r of the sample e.g. Superdex™, Superose™, Sephadex™, Sepharose™, Sephacryl™,
- Peristaltic pump, column and UV monitor
- Buffer with pH 6–8 and ionic strength >0.1

4. Description

Two Gel Filtration Calibration Kits are supplied for protein molecular weight determinations by gel filtration.

The Low Molecular Weight Gel Filtration Calibration Kit contains 50 mg of each of 4 proteins of molecular weight in the range 13 700–67 000 and 50 mg of Blue Dextran 2000.

The High Molecular Weight Gel Filtration Calibration Kit contains 50 mg of each of 4 proteins of molecular weight in the range 158 000–669 000 and 50 mg of Blue Dextran 2000.

The use of gel filtration chromatography for the determination of the molecular weight and size of proteins is well documented. The technique is based on the well-established ability of gel filtration media, such as Superdex, Superose, Sephadex, Sepharose and Sephacryl to separate molecules according to size. Molecular weight determinations by gel filtration are carried out by comparing some elution volume parameter, such as K_{av} of the substance of interest, with the values obtained for several known calibration standards.

In practice it is found that for homologous series of compounds a sigmoidal relationship exists between their various elution volume parameters and the logarithm of their molecular weights. A calibration curve is prepared by measuring the elution volumes of several standard substances, calculating their corresponding K_{av} values (or similar parameter), and plotting their K_{av} values versus the logarithm of their molecular weight. The molecular weight of an unknown substance can be determined from the calibration curve once its K_{av} value is calculated from its measured elution volume. For accurate determination of molecular weight, the calibration standards must have the same relationship between molecular weight and molecular size as the substance of interest. GE Healthcare Calibration Kits provide highly purified, well-characterized, globular protein standards for protein molecular weight determination.

5. Critical parameters

- Select gel with suitable fractionation range for sample
- Select buffer with pH 6–8 and ionic strength >0.1
- Follow instructions carefully when packing the column
- Use recommended flow rate
- Follow instructions carefully when applying sample

6. Protocol

6.1. Preparation of calibration kit

1. Select a gel so that the expected molecular weight of your sample falls approximately in the middle of the fractionation range for that gel.
2. Prepare the gel in the desired buffer. A buffer with a pH of 6–8 and an ionic strength >0.1 is suggested.
3. Carefully pack, equilibrate and adjust the flow rate of a column following the proper procedure.
4. Prepare a fresh solution of Blue Dextran 2 000 (1.0 mg/ml) in the eluent buffer. Apply a sample to the column [sample size=1–2% of the total gel bed volume (V_t)] to determine the void volume (V_0), and check the column packing.
5. Choose the proper Calibration Kit proteins. Include Calibration Kit proteins of a higher molecular weight and of a lower molecular weight than that of the sample.
6. Dissolve the proper combination of Calibration kit proteins in the eluent buffer. The concentration of each protein should be between 5 to 20 mg/ml (except ferritin 1 mg/ml).
7. Apply Calibration Kit proteins to the column, preferably via a 3-way valve and a flow adaptor. The volume of the calibration solution should be 1–2% of the total gel bed volume (V_t).
8. From a UV chromatogram, determine the elution volumes (V_e) for the Calibration Kit proteins by measuring the volume of the eluent from the point of application to the centre of the elution peak.
9. Calculate the K_{av} values for the Calibration Kit proteins and prepare a calibration curve of K_{av} versus log molecular weight.
10. Apply the unknown sample (volume 1–2% of V_t) and determine the elution volume (V_e) of the compound of interest.

11. Calculate the corresponding K_{av} for the component of interest and determine its molecular weight from the calibration curve prepared using the Calibration Kit proteins.

6.2. Gel and calibration kit selection

Fig.1 illustrates the molecular weight determination ranges for Sephadex, Sepharose and Sephacryl. These ranges are based on the linear portion of the calibration curve for these gels.

The relative elution positions of low molecular weight (o) and high molecular weight (•) Calibration Kit proteins are indicated on the bar graphs for the various gels.

Select a gel so that the sample's molecular weight falls approximately in the middle of the gel's molecular weight determination range. Superdex 200 can be used for a preliminary, quick approximation of the sample's molecular weight, if it is totally unknown.

The proper choice of Calibration Kit proteins should include protein standards that are higher and lower in molecular weight than the sample. Therefore, the following recommendations are made on the choice of Calibration Kits:

Calibration Kit(s)	Gel
LMW	Sephadex G-75, G-75 S
LMW	Sephadex G-100, G-100 SF
LMW	Superdex 30 prep grade
LMW	Superdex 75, Superdex 75 prep grade
LMW, HMW	Superdex 200, Superdex 200 prep grade
LMW, HMW	Superose 12, Superose 6
LMW, HMW	Sephacryl S-200 HR, Sephacryl S-300 HR, Sephacryl S-400 HR

Figure 1.

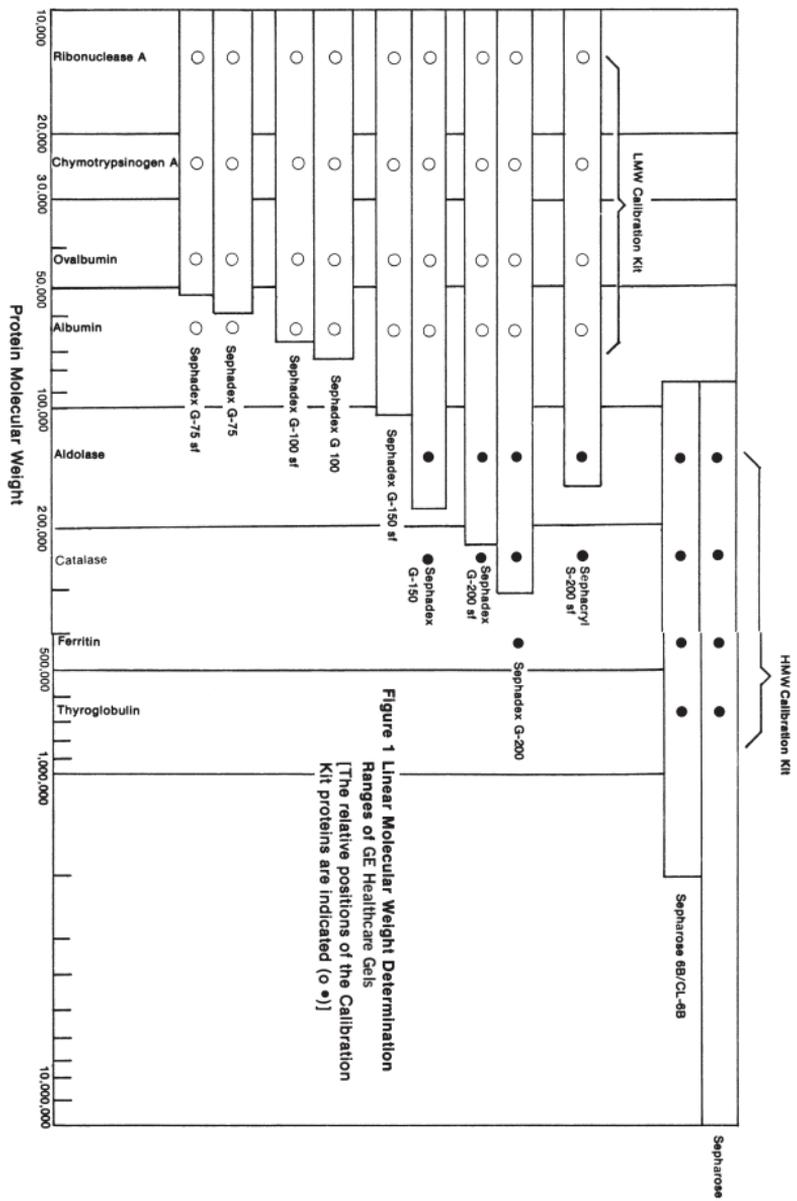


Figure 1 Linear Molecular Weight Determination
Ranges of GE Healthcare Gels
 [The relative positions of the Calibration
 Kit proteins are indicated (o •)]

6.3. Gel preparation

To assure the best results, it is recommended that careful attention be given to gel preparation and column packing. It is suggested that a buffer with a pH of 6–8 and an ionic strength of >0.1 be employed with the Calibration Kit proteins.

Sephadex

1. With gentle mixing, slowly add the required amount of Sephadex (see Table 1) to a volume of buffer equal to three times expected bed volume.
2. Allow the gel to swell for the period of time shown in Table 1. Continuous stirring should be avoided. do not use magnetic stirrer.
3. Resuspend the gel and allow it to settle for approximately 20 minutes.
4. Remove excess buffer and fine particles by suction.
5. Resuspend the gel in a volume of buffer approximately equal to the settled volume of gel.
6. Degas the buffer and gel slurry.
7. The gel slurry should reach the temperature of column operation before bed packing is begun. (The Calibration Kit proteins may be chromatographed at room temperature, however, cold room operation may be desirable if sample proteins are labile at room temperature.)

Table 1. Physical data for GE Healthcare Gels

	Sephadex				Sephacryl S-200 SF		
	G-75	G-100	G-150	G-200	6B	4B	2B
Gel bed volume (ml/g dry weight)	15	20	30	40	Supplied swollen		
Swelling time 20°C (hour)	24	48	72	72	Supplied swollen		
Swelling Time 90°C (hour)	3	5	5	5	Supplied swollen		
Maximum packing pressure (cm of H ₂ O)	160	96	36	16	200	80	40
Recommended operating flow rate (ml/cm ² -hour)*	2–5 (ml/cm ² -hour)						2–30 (ml/cm ² -hour)

* Absolute flow rate = cross sectional flow rate x cross-sectional area of column in cm² (column)

Sephacryl

1. Suspend the pre-swollen gel in buffer equivalent to approximately three times the volume of settled gel.
2. Allow the gel to settle and decant the excess buffer.
3. Repeat the buffer suspension and decanting two more times to allow preliminary equilibration of the gel with the eluent buffer.
4. Resuspend Sephacryl in a volume of buffer approximately 30% in excess of the settled volume of gel.
5. Degas the buffer and gel slurry.

6.4. Column selection

Although molecular weight determination by gel filtration can be conducted in a very simple manner by descending column chromatography, for best results, reliability and convenience, it is recommended that a 2.6 x 40 cm column with two flow adaptors be employed (such as the GE Healthcare K26/70 column with flow

adaptors). Flow adaptors enhance the ease of column operation by allowing simple, quick and reproducible sample application, as well as upward flow elution. A bed length of 40 cm is sufficient for most determinations although longer columns may be employed.

6.5. Bed packing

1. Mount the column and packing reservoir vertically, check for leaks and remove any trapped air from the bed support. A recommended column setup is shown in Figure 2. (p.15)
2. Fill the column with buffer to a height of 5–10 cm and turn off column flow.
3. Suspend swollen gel and carefully pour all the slurry into the gel reservoir. The concentration of slurry should be such that air bubbles which may be formed can readily rise to the surface.
4. Allow the gel to settle for 5 minutes.
5. A) Sephadex and Sepharose - Establish recommended hydrostatic pressure shown in Table 1, then open column outlet. Continue packing until the gel reaches a stable bed height and is equilibrated with the eluent buffer.
B) Sephacryl S-200 Superfine - For best results with Sephacryl, the gel should be packed at a flow rate of about 40 ml/cm²-hour.** Pack the gel using downward elution at a constant flow rate of 40 ml/cm²-hour for approximately 2 hours. Secure a flow adaptor just touching the upper gel surface, and elute upward; maintain the same flow rate for another 2 hours. The column is now ready for upward or downward chromatography at a maximum sustained flow rate of 30 ml/cm²-hour.

For most gel bed lengths, this packing procedure requires the use of a pump for packing and running the columns of Sephacryl because of the large hydrostatic pressure head needed to achieve a 40 ml/cm²-hour flow rate. If a pump is not available, pack the

sephacryl at

>200 cm of H₂O hydrostatic pressure and run the column at a hydrostatic pressure less than packing pressure.

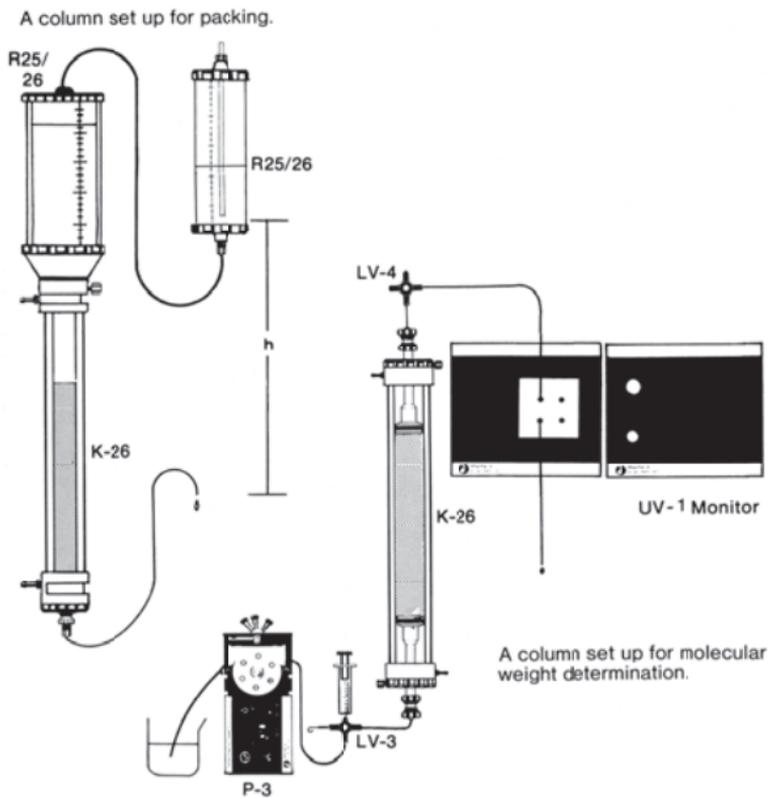
Void volume determination

The elution volume for Blue Dextran 2 000 is equal to the column void volume (V_0). Prepare a fresh solution of Blue Dextran 2 000 (1.0 mg/ml) in the eluent buffer. The rate of solubilization of the Blue Dextran 2 000 may be increased by heating the buffer to 50°C before adding the Blue Dextran 2 000. Also, it is strongly recommended that Blue Dextran 2 000 be run **alone**, not mixed with the Calibration Kit or sample proteins, because of the chance of protein adsorption to the broad fraction of Blue Dextran.

Determine the elution volume (V_e) for a sample of Blue Dextran 2 000 solution according to the procedures described under Sample application and measurement of elution volumes. The sample volume of Blue Dextran should be 1% of the total gel bed volume (V_t). The elution of Blue Dextran can be conveniently monitored at wavelengths of 254, 280 or 620 nm.

**Column flow rate=cross-sectional flow rate x column cross-sectional area in cm²

Figure 2.



6.6. Selection and preparation of calibration standards

In order that the Calibration Kit proteins be sufficiently resolved, it is necessary that the appropriate protein mixtures be employed. In addition, the sample volume and protein concentration must be appropriate for the column size and sensitivity of the detection equipment.

When calibrating a column, it is recommended that the calibration proteins be run in at least two separate groups to ensure enough resolution of their peaks for accurate elution volume measurement. Groups of Calibration Kit proteins that can be mixed together for a single chromatographic run on the various gels using a column of at least 40 cm in length are listed below.

For calibration of:	Run I	Run II
Sephadex G-75, G-75 SF	ribonuclease A	chymotrypsinogen A
Sephadex G-100, G-100 SF	and ovalbumin	and albumin
Superdex 75		
Superdex 200	ribonuclease A, ovalbumin, aldolase and ferritin	chymotrypsinogen A albumin and catalase

For calibration of:	Run I	Run II
Superose 6	aldolase, ferritin and albumin	catalase thyroglobulin

Note: Recommended calibration proteins that will not be on the linear portion of the K_{av} vs. log molecular weight curve is:

albumin for Sephadex G-75, G-75 SF

Each Calibration Kit protein should be dissolved to a concentration of 5 to 20 mg/ml with the exception of ferritin, which needs to be dissolved to a concentration of only 1 mg/ml (since it has a much higher extinction coefficient at 280 nm than the other Calibration Kit proteins). At 5 mg/ml concentration and a sample size of 1% of V_t , the Calibration Kit proteins will have a peak absorption of approximately 0.3 O.D. units. When making the calibration solutions containing aldolase, catalase and ferritin, remember to take into consideration

that part of the solid material present is sucrose or mannitol.

To dissolve the Calibration Kit proteins:

1. Add a measured volume of buffer to the appropriate preweighed protein mixture.
2. Allow the sample to stand for approximately 10 minutes.
3. Mix gently with a stirring rod. Do not heat or mix vigorously.

The resulting protein solutions should be completely dissolved and free of insoluble materials.

The sample volume applied to the column should be 1–2 ml. This represents a sample approximately 1% of the total gel bed volume (V_t) for a 2.6 x 40 cm gel bed.

6.7. Sample application

Sample application is a critical step in liquid chromatography because unnecessary sample dilution and uneven penetration into the bed can result in zone broadening, adversely affecting resolution.

For ascending elution of column using flow adaptors
(The recommended procedure)

1. Vent any air from the 3-way valve using a syringe filled with eluent buffer.
2. Draw the sample into the syringe and attach the syringe to the valve being careful to exclude air bubbles.
3. Prepare the column monitoring and fraction collecting equipment. (see Figure 2).
4. Apply the sample manually or with syringe pump at a flow rate one-half that used for column elution.
5. (Optional) Apply 2 ml 10% sucrose in buffer immediately after sample to aid in sharp sample application.
6. Position the 3-way valve to start column elution, and adjust flow rate to agree with the value recommended in Table 1.

6.8. Measurement of elution volume (V_e)

For reproducible results, it is necessary that the elution volume of each component be determined as accurately as possible. If a device such as a continuously monitoring UV photometer or differential refractometer is used, the elution volume must be correlated to the elution profile. This can be achieved by automatic or manual indexing of the elution profile on a time, volume or weight basis. A burette for measuring cumulative eluted volume with manual indexing of the eluted profile may also be used. The use of a pump to maintain a constant flow rate is advantageous. It is advisable to check the accuracy and reproducibility of whatever collection method is selected.

Determination of the elution volumes (V_e) for the Calibration Kit proteins from the elution profile is illustrated in Figure 3 (p.20).

The elution volume is measured from the start of the sample application to the centre of the elution peak, as determined by the intersection of the two tangents drawn to the sides of the peak. [When the sample is Blue Dextran 2 000, V_e is then the void volume(V_0).]

6.9. Preparation of calibration curve

A molecular weight calibration curve defines the relationship between the elution volumes of a set of standards and the logarithm of their respective molecular weights. Various elution parameters, such as V_e , V_e/V_0 , K_d , erf^{-1} of K_d , and K_{AV} have been used in the literature for the preparation of calibration curves. The use of K_{AV} is recommended in lieu of other elution parameters since: 1) it is less sensitive to errors which may be introduced as a result of variations in column preparation and column dimensions, 2) it does not require the unreliable determination of the gel internal volume (V_i) as is required with K_d , and 3) it does not require accessory mathematical tables as is required for the calculation of erf^{-1} of K_d .

The recommended procedure is:

1. Calculate K_{AV} values for each protein using the equation

$$K_{AV} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e = elution volume for the protein

V_o = column void volume = elution volume for Blue Dextran 2 000

V_t = total bed volume

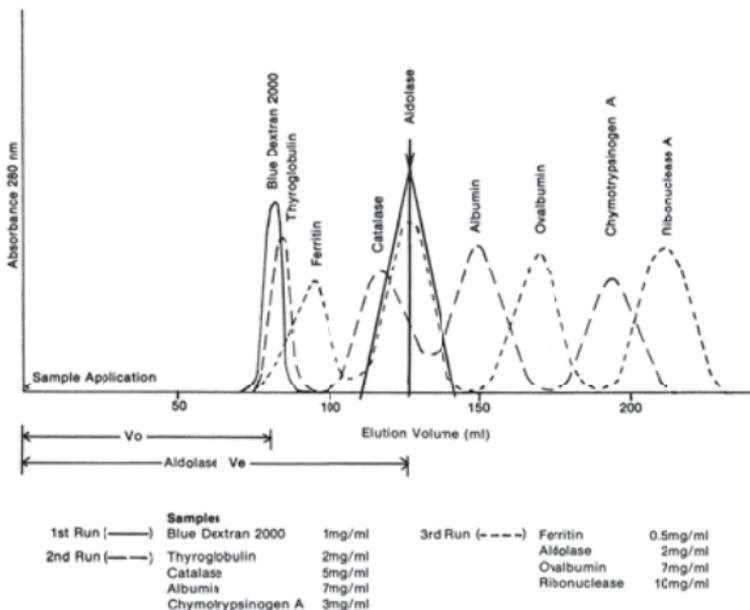
2. Using semilogarithmic graph paper, plot the K_{AV} value for each protein standard (on the linear scale) against the corresponding molecular weight (on the logarithmic scale).
3. Draw the straight line which best fits the points on the graph. Note that proteins of molecular weights which approach the limits of the fractionation range for a given Sephadex will not have K_{AV} values which fall on the linear portion of the calibration curve. Figures 4, 5 and 6 show examples of calibration curves which have been obtained using the Calibration Kit proteins on Sephadex G-75 Superfine (Figure 4, p.22) Sephadex G-200 (Figure 5, p.23) and Sepharose CL-6B (Figure 6, p.24).

Chromatography of proteins of unknown molecular weight

1. Adjust the concentration of the sample to allow detection by a convenient method such as UV absorption or enzymatic activity, taking into consideration that a sample 1% of V_t will be diluted 8-15 fold during chromatography.
2. The sample volume should be equal to that used for calibration mixtures.
3. If necessary, centrifuge the sample to obtain a clear solution.
4. Apply the sample solution and elute according to the procedure used for the Calibration Kit proteins.

Figure 3. Elution profiles for Calibration Kit proteins on Sephadex G-200

Column: K2670 with flow adaptors
 Bed Dimensions: 2.6 X 49.7 cm
 $V_0 = 81$ ml
 Eluent: 0.05M potassium phosphate pH 7.5
 containing 0.1M NaCl and 0.02% NaN_3
 Sample Volume for each run = 1.0 ml
 Flow Rate: 18 ml/hr.



6.10. Molecular weight distribution

1. Measure the elution volume (V_e) of the protein(s) of interest.
2. Calculate the K_{AV} for the protein(s) of interest.
3. Locate the point on the calibration curve which corresponds to the K_{AV} (or other elution volume parameter) value for the protein of unknown molecular weight. The value on the logarithmic scale which corresponds to this point is the estimated molecular weight of the protein.

In order to achieve the greatest precision and accuracy in the determination of molecular weights, it is important that each column be calibrated with the standard proteins and that the corresponding calibration curves be established. Errors in molecular weight estimations may occur for several reasons if each column is not calibrated. Variations in pore size distribution of gel preparations, column packing and changes in composition, ionic strength or pH of eluents may slightly alter elution parameters and consequently affect the slope or position of the calibration curve.

Molecular weight determinations by the above procedure assume the same relationship between molecular size and molecular weight for all unknowns and standards. All of the Calibration Kit proteins are, to a good approximation, globular in shape. The molecular weights of glycoproteins, lipoproteins, non-globular proteins, or other polymers may not correlate well to the calibration curves established for globular proteins by the Calibration Kit proteins. For such compounds, useful information can be obtained by relating their elution volume data to a molecular size parameter, such as Stokes' radius (R_s), rather than to molecular weight values. Plots of $\sqrt{-\log(K_{av})}$ vs. R_s have been used successfully to determine the Stokes' radius of proteins.

7. Typical results

Figure 4. Calibration curve using the Low molecular Weight Gel Filtration Calibration Kit on Sephadex G-75 Superfine

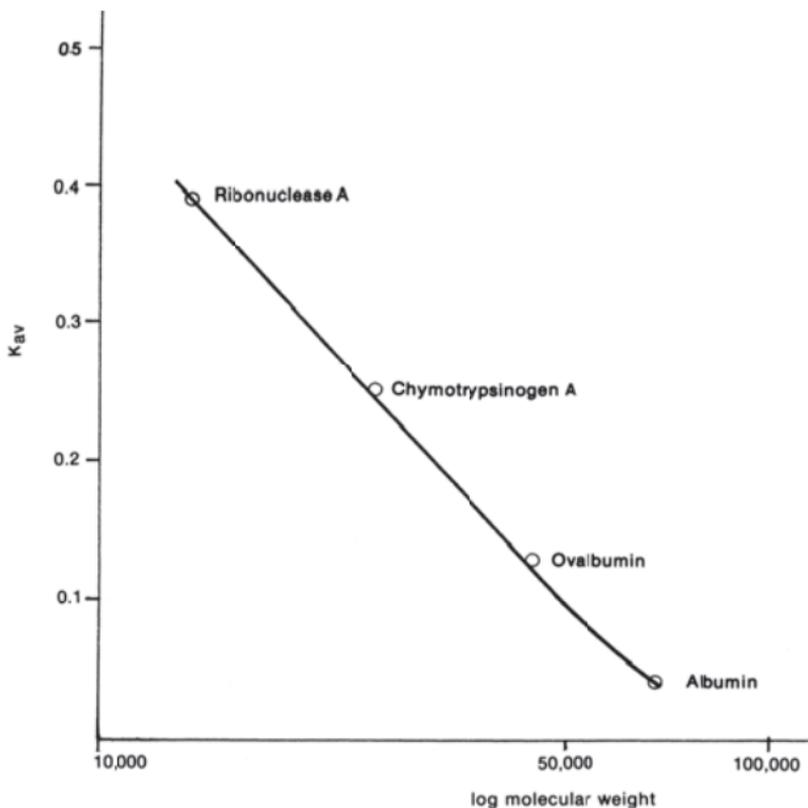


Figure 5. Calibration curve using both Low(o) and High (•) Molecular Weight Gel Filtration Calibration Kits on Sephadex G-200

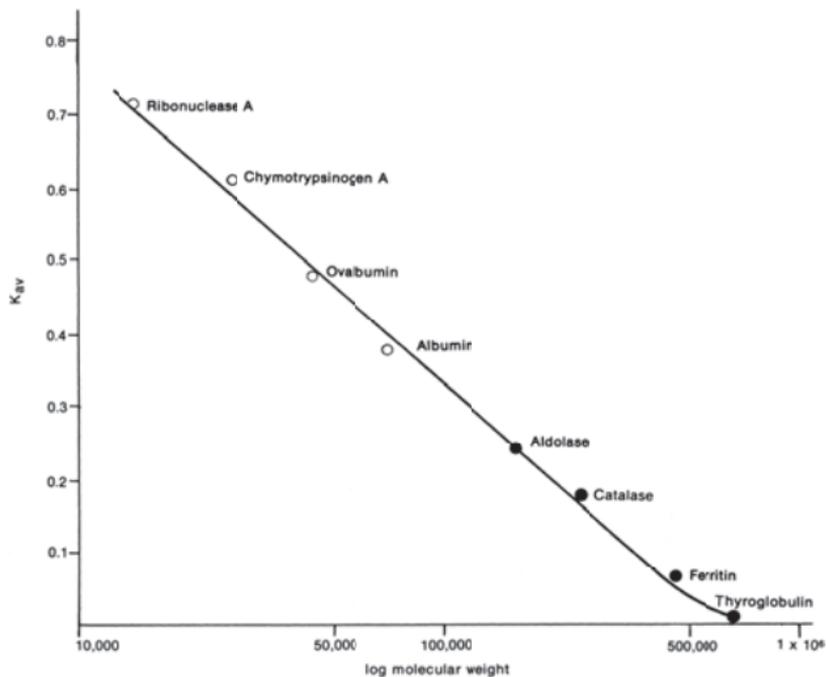
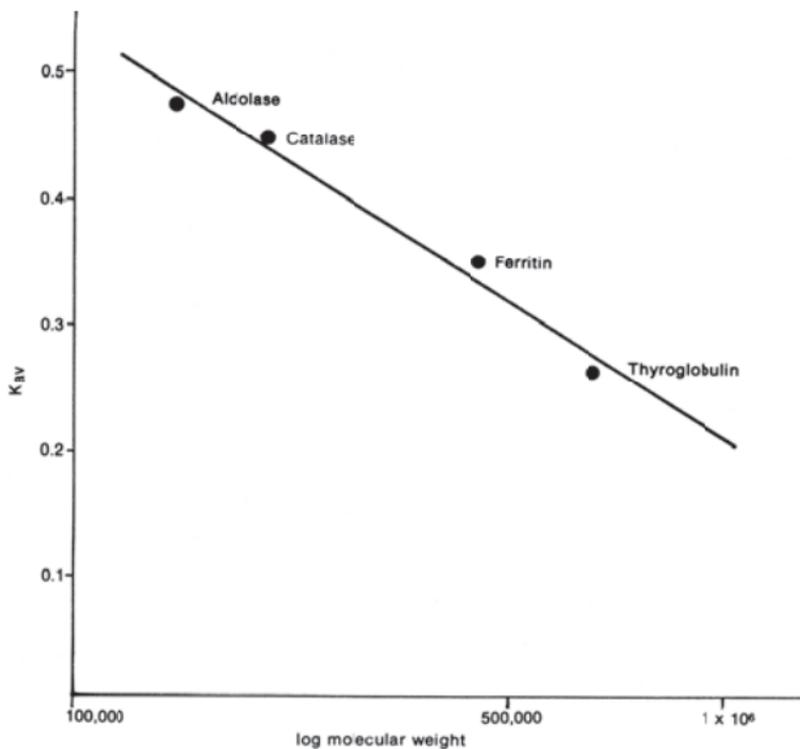


Figure 6. Calibration curve using the High Molecular Weight Gel Filtration Calibration Kit on Sepharose CL-6B



8. Additional information

8.1. Important notes

- **The use of the Calibration Kits with denaturing solvents**

The molecular weight determination ranges given in Table 1 are for globular proteins in their native conformations. The use of denaturing agents like Sodium Dodecyl Sulphate, chaotropic salts and Guanidine Hydrochloride and Hydrogen bond disrupting agents, such as Urea, may alter the molecular conformation of proteins often greatly increasing their hydrodynamic volumes. Since separations by gel filtration are based on molecular size, the molecular weight determination ranges change when the proteins assume extended conformations.

In fact, the gel with the most useful molecular weight determination range and flow properties in solvents where proteins completely denatured is Sepharose CL-6B (exclusion limit is approximately 120 000 for completely denatured proteins). The Low Molecular Calibration Kit is suitable for the calibration of columns in denaturing solvents. These proteins all comprise a single polypeptide chain, therefore, their molecular weights do not change when they are exposed to denaturants (although their Stokes' Radii do change).

- **Aggregation of calibration kit proteins**

The ribonuclease A, albumin, aldolase, ferritin and thyroglobulin standards may contain small amounts of apparent aggregates which elute in the void volume or slightly before true peak. Tangents drawn to such peaks should neglect shoulders due to aggregates.

- **Thin-layer gel filtration on Sephadex**

Sephadex thin-layer gel filtration can also be used for molecular weight determinations with the Calibration Kit when it is desirable

to use small amounts of protein. The standard proteins are spotted on thin-layer plates which have been spread with the appropriate Sephadex. At the end of the experiment, the positions of the proteins are determined by staining, enzyme activity, etc. The migration distances of the protein standards are plotted against the logarithms of their molecular weights to obtain the calibration curve. The unknown molecular weight can then be read directly from the calibration curve.

- **Electrophoresis Calibration Kits**

The GE Healthcare High molecular Weight and Low Molecular Weight Gel Filtration Calibration kits contain protein standards for use in gel filtration chromatography only. Also available from GE Healthcare are Calibration Kits containing protein standards for molecular weight determination by polyacrylamide gel electrophoresis.

9. Related products

Gel Filtration Columns and
Media Selection Guide

18-1124-19

Gel Filtration:
Principles & Methods

18-1022-18

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