Size-exclusion chromatography in an analytical perspective

Lars Hagel

R&D Department, Pharmacia LKB Biotechnology AB, S-751 82 Uppsala (Sweden)

ABSTRACT

Modern media for size-exclusion chromatography combine maximum selectivity with high plate count to resolve solutes differing in molecular mass by as little as 20%. Separations of solutes differing in molecular mass by only 10% will be very hard to accomplish even for rod-shaped molecules. The reduced column lengths and porosity of small-particle-sized media unfortunately reduces the inherent resolving power to the same order as traditional columns but with a substantial reduction in analysis time. The use of well-calibrated columns for obtaining estimates of molecular mass yields a precision close to that of absolute methods. The simplicity and accuracy of the integral calibration method using a sample of broad molecular mass distribution makes this procedure very suitable for manual calibration of size-exclusion columns.

The objective of analytical size-exclusion chromatography is to separate components of different size well enough to permit their quantitative and/or qualitative determination. The purpose of this communication is to review the present status of size-exclusion chromatography as a tool for analytical characterization of macromolecules.

SEPARATION OF COMPONENTS

Separation is achieved by passing the sample through a porous support. The degree of separation may be quantified by the resolution between peaks. The resolution is a function of the distance between peaks and the width of the peaks. The peak-to-peak distance, or selectivity, is determined by the number and dimension(s) of the pores and is thus set by the characteristics of the gel medium. The width of peaks is influenced by the particle size of the medium. The resolution is also affected by experimental parameters such as column length, eluent velocity and solvent viscosity.

The maximum selectivity of size-exclusion gel media is obtained by using single pore size supports. For such a support it may be shown that the separation range covers approximately one decade in solute radius [1]. Owing to the different relationships between molecular mass and size for solutes of different shape the selectivity for one decade in size will correspond to one, two and three decades in mass for rods, flexible coils and spheres, respectively [2]. Some traditional media for aqueous SEC display such high selectivity (*e.g.* Sephadex and Bio-Gel). Porous glass also yields high selectivity owing to the narrow pore size distribution introduced by the manufacturing process [1]. One example of a modern medium constructed to give high selectivity is porous silica microspheres, where the pore dimension is given by the space between solid silica sub-microspheres of uniform size [3]. A novel medium for aqueous size-exclusion chromatography combines the rigidity of a macroreticular gel with the selectivity of the microreticular gel by the incorporation of dextran into a cross-linked agarose skeleton. The selectivity, as shown in Fig. 1, is close to maximal. This type of medium is preferentially used for separations where differences in solute size are minute. One such example is the analysis of
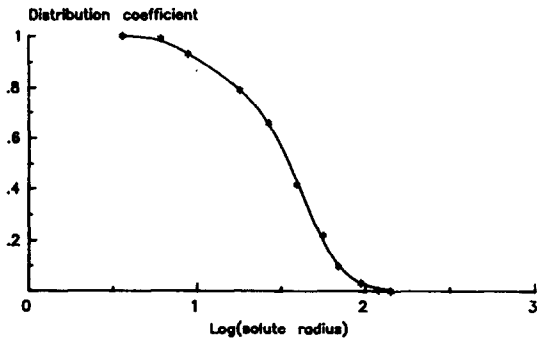
Fig. 1. Selectivity of Superdex 200 for dextrans. Narrow fractions of dextran were run through a column of Superdex 200 prep grade, 62 × 2.6 cm I.D. using a fast protein liquid chromatography system. The molecular mass corresponding to peak elution volume was converted to solute radius through $R_h = 0.271M^{0.498}$ (ref. 1). (Raw data used by courtesy of ref. 4.)

multimeric forms of important biomacro-molecules, as illustrated by Fig. 2.

The theoretical limit of resolution, R_s, in size-exclusion chromatography may be inferred from the resolution equation [5]:

$$R_s = 1/4 \cdot \log (M_1/M_2) \cdot N^{1/2}$$

$$\cdot \, [-d(K_D)/d(\log M)]/(V_0/V_p + K_D) \qquad (1)$$

where M_1 is the molecular mass of species 1, K_D

is the distribution coefficient, $d(K_D)/d(\log M)$ is the molecular mass selectivity of the medium, V_0 is the void volume of the column, V_p is the pore volume of the gel bed and N is the average plate number of the two solutes. The plate number of the solutes may be calculated by using the assumption that the separation is carried out at optimum eluent velocity where peak widths are fairly constant [6]. Inserting $\sigma = \sigma_t$ and $V_R = V_t[1 + V_p/V_t(K_D - 1)]$ in the formula $N = (V_R/\sigma)^2$ and realizing that the maximal plate count is obtained for small solutes, i.e. $N_{max} = (V_t/\sigma_t)^2$, eqn. 1 can be rearranged to:

$$\log (M_1/M_2) = 4R_s(V_0/V_p + 1)(N_{max})^{-1/2}$$

$$\cdot \, 1/[-d(K_D)/d(\log M)] \qquad (2)$$

where the first term accounts for the required resolution, the second term is a function of the pore volume of the support, the third term expresses the maximal plate count of the column and the last term reflects the pore size distribution of the media.

The maximal size selectivity of a support is obtained at the inflexion point of the selectivity curve, which is given by the distribution coefficient as a function of the logarithm of solute radius, R. The inflexion point is obtained at $R = r/2$, where r is the pore radius of the support [1]. Using a cylindrical pore model it can be
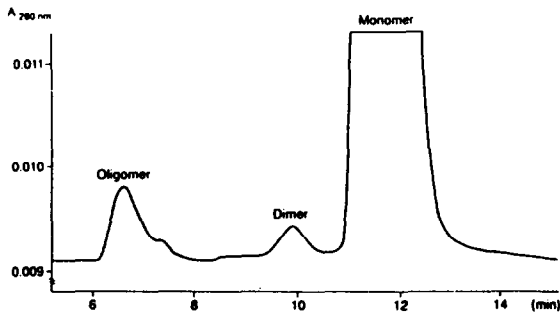

Fig. 2. Analytical size-exclusion chromatography for determination of aggregates in preparations of recombinant human growth hormone (rhGH). The sample, 0.05 ml of rhGH, was applied to Superdex 75 HR 10/30, 30 × 1 cm I.D., and eluted at 1 ml/min with 0.05 M sodium dihydrogenphosphate in 0.1 M sodium sulphate, pH 7.3. An enlarged section of the original chromatogram is shown. (Courtesy of B. Pavlu, Kabi-Pharmacia Peptide Hormones, and H. Lundström, Pharmacia.)

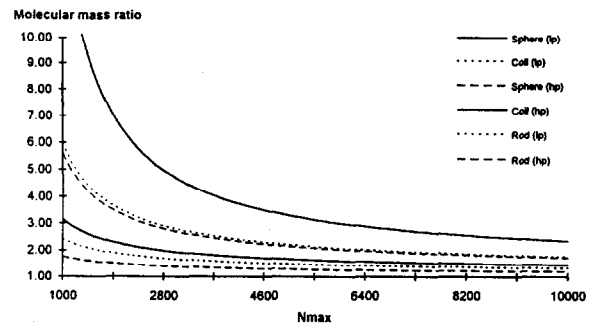Fig. 3. Resolvability of size-exclusion chromatography. The molecular mass ratio needed for complete resolution of solutes, i.e. $R_s = 1.5$ as a function of column plate number is given for various solute shapes and column permeabilities. Curves plotted in order as given by the explanation. Permeability, V_p/V_0, is for low-porous media (lp) = 0.75 and high-porous media (hp) = 2.0. Calculated from eqn. 2.

shown that the inflexion point for a single pore size support is given by [1]:

$$|d(K_D)/d(\log R)|_{max} = \ln(10)/2 = 1.15 \qquad (3)$$

In terms of molecular mass the maximal selectivity will equal 1.15 for rods, 1.15/2 for flexible coils and 1.15/3 for spheres. The selectivity for proteins is frequently reported to exceed 0.38. This is probably because the shape of globular proteins is ellipsoidal and may not be approximated by spheres.

The resolvability, i.e. the ability to completely resolve solutes of different sizes, as a function of plate number of different solute shapes and media pore volumes is shown in Figs. 3 and 4. As illustrated in Fig. 3 most media, including traditional ones, will separate solutes differing a decade in molecular mass. Also noticeable is the large influence of the pore fraction, V_p/V_0, of the media on the resolvability. The maximal resolvability that can be expected from size-exclusion chromatography is shown by Fig. 4. Whereas a separation of dimer from monomer may be expected from a 30-cm column packed with a 10-μm bead, typically yielding $N_{max} = 10000$ (see Fig. 2), separation of proteins differing in molecular mass by less than 20% will be difficult also with columns of extreme plate numbers. The limit for rod-shaped molecules, such as DNA, seems to be in the vicinity of 10%. Thus, if the molecular mass of solutes of similar shape differs by less than 10%, separation by some principle other than size should be explored.
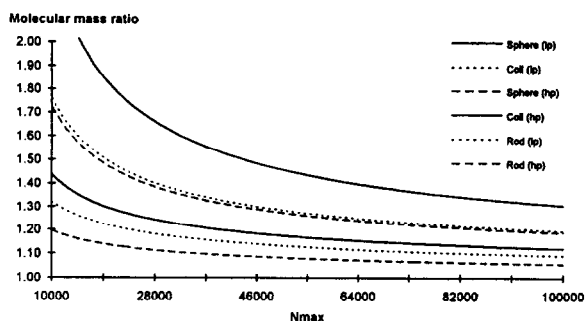

Fig. 4. Resolvability of size-exclusion chromatography using columns of extreme plate counts. Conditions as given in the legend to Fig. 3.

It is important to note that high selectivity yields a low separation range and this is not always desired. For instance, when analysing molecular mass distributions, a separation range covering the complete size range of solute species is required to yield accurate information. It may also be noted that the decrease in column length and pore fraction of modern microparticulate media for size-exclusion chromatography results in lower peak capacity, i.e. resolvability, than expected from plate count data. A comparison of peak capacities of different media–column combinations shows a maximum of thirteen completely resolved peaks for both modern and traditional columns [6]. However, the latter type of columns may be operated very fast without too much loss in resolvability as compared with traditional media.

DETERMINATION OF SOLUTE SIZE

The size of solutes may be determined either by using a calibrated system or by employing a size-specific detector. In the first case the column is calibrated with the aid of standards of known size and preferably of the same geometry as the solute to be characterized. Such systems have been utilized for many years for the characterization of molecular mass distributions of polymers. The systems have proven to be very reliable and the major contribution to variability stems from inaccuracy in the data for the calibration samples [7]. The accuracy of the calibration is verified by running reference samples. The reference materials are characterized by an absolute method, e.g. light scattering, and the small variability of this technique over an extended period of time is illustrated in Fig. 5. Corresponding data for some different calibrated column systems are shown in Fig. 6. It can be concluded that the variability of the two methods is of the same order of magnitude, i.e. displaying a relative standard deviation of approximately 2%, illustrating that size-exclusion chromatography yields data of high precision.

The calibration procedure of the column may be very tedious, involving running a large number of samples, plotting an estimate of the molecular mass versus elution volume and then
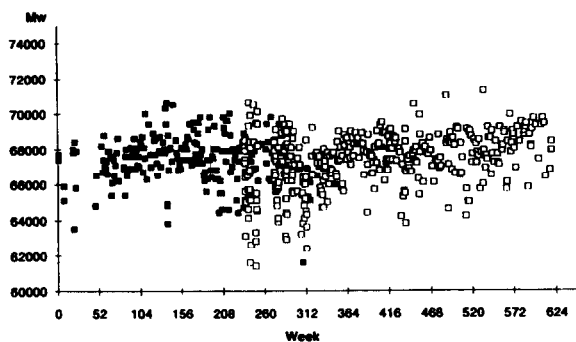
Fig. 5. Weight-average molecular mass, M_w, for a control dextran as determined by light scattering over a period of 12 years. Filled squares represent data obtained with Sofica 2; number of data, $n = 223$, mean value, $x = 67431$, and standard deviation, $s = 1337$. Open squares represents data obtained with KMX-6; $n = 361$, $x = 67253$ and $s = 1621$. (Raw data used by courtesy of ref. 8.)

fitting a high-degree polynomial or another function to the data points. The procedure requires the use of a computer for convenient handling of the calculations. The use of high-degree polynomials for curve fitting may introduce unexpected variations of the curve, especially at the extremes of the calibration range.

Recently, Hagel and Andersson [9] illustrated the use of the integral calibration method for a rapid, simple and accurate calibration of columns in solute size. In this method, the entire distribution is used for calibration, thus providing a very large number of data points, which obviates
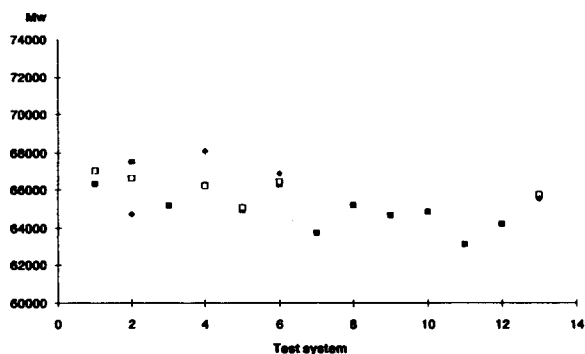

Fig. 6. Weight-average molecular mass, M_w, for a control dextran as determined by size-exclusion chromatography. The same sample as used in Fig. 5 was analysed using different gels and columns yielding $x = 65824$, $s = 1337$ with $n = 25$. (Raw data used by courtesy of ref. 8.)

the need for any curve-fitting procedure. Furthermore, by selecting a calibration substance of sufficiently broad molecular mass distribution, the separation range of interest may be calibrated from a single run. The prerequisite is that the molecular mass distribution of the calibration substance must be accurately known. The method, as applied for determination of solute size of proteins using dextran as calibration sample, is illustrated in Fig. 7. The sample was run on the column to be calibrated (Superose 6), and the cumulative weight fraction of eluted material was calculated simply by reading the response from the recorder chart at successive intervals and normalizing the data, as shown in Fig. 7a. The molecular mass corresponding to each slice of cumulative area was calculated from the known molecular mass distribution of the sample (Fig. 7b) and plotted as a function of retention volume (Fig. 7c). Finally, the molecular mass was transferred to viscosity (hydrodynamic) radius by using the relationship $R_h = 0.271 M^{0.498}$ (ref. 1), to yield the final calibration curve in solute size (Fig. 7d). This type of calibration is useful for estimation of size of globular proteins [10–12], and the applicability is illustrated by the evaluation of apparent size of some proteins as compared with literature data in Table I. The accuracy of this simple procedure is in this case better than 5%. Furthermore, peak molecular mass for narrow dextran fractions is in excellent agreement with the nominal data given by the manufacturer. Thus, for many investigations the very simple and time-saving integral calibration method yields sufficient accuracy and precision for the determination of molecular mass of dextran and apparent solute size of globular proteins. As pointed out by Yau et al. [13], the method yields less precise calibration in the extremes of the size distribution of the calibration sample owing to column band broadening and experimental imprecision. However, by choosing a sample of broad size distribution a sufficient size range is easily covered, i.e. the dextran used in this case yields a calibration range form 2.7 to 11.5 nm.

In case the column is used for obtaining molecular mass distributions, the band broadening of the column will influence the experimentally determined estimates. Models for correc-
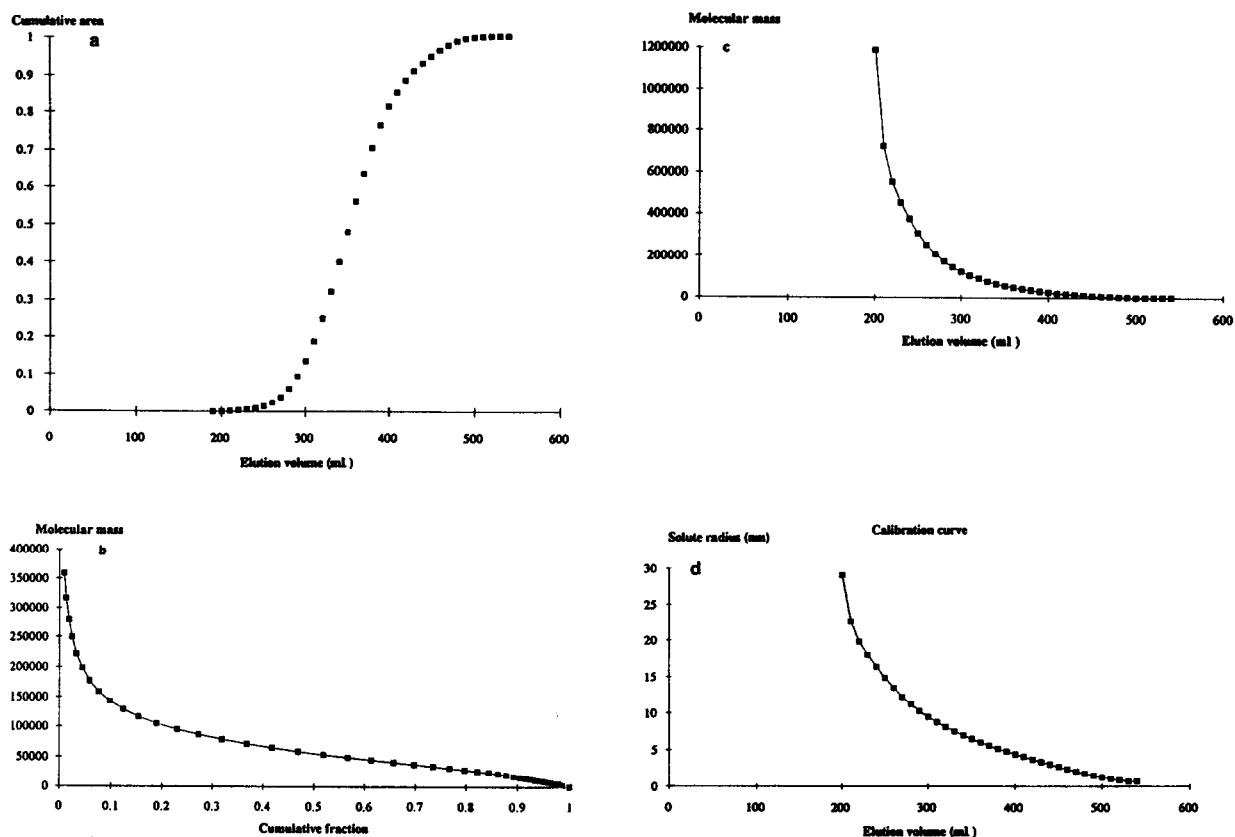
Fig. 7. Illustration of the integral calibration method using dextran of broad molecular mass distribution. (a) The dextran sample with $M_w = 70\,450$ and number-average molecular mass, $M_n = 27\,650$ was chromatographed on Superose 6 packed in a 315 × 16 mm I.D. column and eluted at 1 ml/min using 0.25 M sodium chloride. The cumulative area was calculated from the peak height at 36 successive elution volumes and plotted. (b) The molecular mass distribution as obtained using a carefully calibrated gel filtration column. (c) The resulting calibration curve from data in Fig. 7a and b. (d) The calibration curve in viscosity radius of dextran using the relationship $R_h = 0.271 \cdot M^{0.498}$ (ref. 1). From ref. 9.

tion of band broadening often assume that the extent of band broadening is constant over the entire separation range. However, in reality band broadening may vary quite considerably, especially at high eluent velocity, where non-equilibrium effects cause large broadening of solute bands of higher molecular mass [6]. Application of band-broadening corrections have therefore been questioned, and some authors have found that correction procedures introduce errors rather than eliminate them [14,15]. The band-broadening effect may be minimized by operating the column at optimal eluent velocity where band broadening is minimal [5]. Using the equations proposed by Yau et al. [16], it may be calculated that the error in experimental molecu-

lar mass is +1 to +2% for the weight average and −1 to −2% for the number average when analysing samples on 10-, 30- or 100-μm medium packed in 25-, 50- or 100-cm-long columns, respectively (the larger errors are obtained for the larger particle sizes). The interstitial eluent velocity required may be estimated from $u = 65K_D D_m/d_p$, where D_m is the diffusion coefficient of the solute and d_p is the average particle size of the gel medium [5]. Band broadening will influence the molecular mass estimates, as outlined above, if the column is calibrated using the molecular mass corresponding to the peak apex (i.e. since the position of the peak is not affected by the band broadening). The effect of band broadening may in some cases be compensated

TABLE I

ESTIMATION OF MOLECULAR MASS AND SOLUTE RADIUS

Note that data in parentheses are for solutes eluted outside the recommended calibration range (see text for explanation).

Solute	Measured		Nominal		
	M_{dextran}	$R_{\mathrm{h}}(\mathrm{nm})^a$	M_{peak} [b]	$R_{\mathrm{St}}(\mathrm{nm})^c$	$R_{\mathrm{h}}(\mathrm{nm})^d$
Dextran	195 300	11.7	196 300		
Dextran	191 500	11.6	196 300		
Dextran	66 880	6.8	66 700		
Dextran	19 310	3.7	21 400		
Dextran	(7230	2.3)	9980		
Thyroglobulin	102 619	8.4		8.1, 8.5	8.3
Ferritin	47 890	5.8		5.9	6.1
Bovine serum albumin	19 305	3.7		3.6	3.6
Myoglobin	(5914	2.0)			2.0
Cytochrome c	(4452	1.8)		1.7	1.6

[a] Measured $R_{\mathrm{h}} = 0.271 \cdot (M_{\mathrm{dextran}})^{0.498}$, from ref. 1.
[b] Nominal data from the manufacturer's literature for dextrans (Pharmacosmos).
[c] Stokes radii from ref. 10.
[d] Viscosity radii from ref. 11.

for in the calibration procedure by using an iterative procedure, such as the one described by Nilsson and Nilsson [17], where the calibration is adjusted to yield conventionally true values of weight-average molar mass. For samples of similar polydispersity a correct estimate of the weight average will then be obtained. However, the experimental number-average molecular mass will, in theory, always be smaller than the true value. In both cases it may be expected that the molecular mass distribution will be somewhat broader than the true distribution. In most cases the effect from band broadening will be very small, especially for samples of large polydispersity [15], as also illustrated by the accuracy of the integral calibration procedure in Table I.

The use of size-sensitive detectors coupled on-line with size-exclusion columns has gained increased popularity. In this case the column is solely used for fractionation of the solutes into sufficiently narrow fraction slices for the operation of the detectors. Advantages are that column calibration is not necessary for most studies and that useful information is obtained even in the case of non-ideal size-exclusion chromatography. Detection principles used are based upon classical principles such as light scattering and viscosimetry and may be regarded as a refinement of earlier used batch procedures. Of these principles, the use of light scattering at several angles using laser light sources seems to be very promising for yielding data related to solute size and conformation. The high sensitivity for solutes of large size also makes light-scattering detectors suitable for the detection of very small amounts of aggregates in samples for which detectors ordinarily used, *e.g.* UV or refractive index, show low response. A third principle for determination of molecular mass that is becoming feasible is the interfacing of microcolumns for size-exclusion chromatography with mass spectrometers. However, most of these detectors are rather sophisticated and only suitable for research purposes, and there is still a need for inexpensive and user-friendly detectors suitable for routine laboratory use.

CONCLUSIONS

Resolvability of size-exclusion chromatography seems to be close to 20% with respect to difference in molecular mass of spherical solutes

and somewhat better, *i.e.* down to 10% difference in molecular mass, for rod-shaped molecules.

Use of multiple detection principles on-line with the column yields valuable information about solute size, shape and branching. Detectors suitable for routine purposes will be very useful for detection of aggregates and also for revealing solute–matrix interactions.

The traditional way of working, through use of calibrated columns for determination of apparent size, is still dominant and my be employed also by laboratories lacking facilities for data acquisition and computing by using the simple integral calibration procedure with broad standard(s) of known molecular mass distribution(s).

ACKNOWLEDGEMENT

APPENDIX

Derivation of eqn. 2 from eqn. 1 is done in the following way:

$$R_s = 1/4 \cdot \log{(M_1/M_2)} \cdot N^{1/2}$$
$$\cdot [-\mathrm{d}(K_\mathrm{D})/\mathrm{d}(\log M)]/(V_0/V_\mathrm{p} + K_\mathrm{D}) \qquad (1)$$

Using the following relationships;

$N = (V_\mathrm{R}/\sigma)^2$ (according to the definition of N)

$\sigma = \sigma_\mathrm{t}$ (at optimal conditions, see ref. 6)

$$V_\mathrm{R} = V_0 + K_\mathrm{D}V_\mathrm{p} = V_\mathrm{t}(V_0 + K_\mathrm{D}V_\mathrm{p})/V_\mathrm{t}$$
$$= V_\mathrm{t}(V_0 + K_\mathrm{D}V_\mathrm{p})/(V_0 + V_\mathrm{p})$$
$$= V_\mathrm{t}(V_0/V_\mathrm{p} + K_\mathrm{D})/(V_0/V_\mathrm{p} + 1)$$

yields

$$N^{1/2} = (V_\mathrm{R}/\sigma_\mathrm{t}) = (V_\mathrm{t}/\sigma_\mathrm{t})(V_0/V_\mathrm{p} + K_\mathrm{D})/(V_0/V_\mathrm{p} + 1)$$
$$= (N_\mathrm{max})^{1/2} \cdot (V_0/V_\mathrm{p} + K_\mathrm{D})/(V_0/V_\mathrm{p} + 1)$$

where $N_\mathrm{max} = (V_\mathrm{t}/\sigma_\mathrm{t})^2$ is the maximal plate count for a small solute (*i.e.* the nominal plate count

stated by the manufacturer). Eqn. 1 is thus given by:

$$R_s = 1/4 \cdot \log{(M_1/M_2)} \cdot (N_\mathrm{max})^{1/2}$$
$$\cdot (V_0/V_\mathrm{p} + K_\mathrm{D})/(V_0/V_\mathrm{p} + 1)$$
$$\cdot [-\mathrm{d}(K_\mathrm{D})/\mathrm{d}(\log M)]/(V_0/V_\mathrm{p} + K_\mathrm{D})$$
$$= 1/4 \cdot \log{(M_1/M_2)} \cdot (N_\mathrm{max})^{1/2}$$
$$\cdot 1/(V_0/V_\mathrm{p} + 1)[-\mathrm{d}(K_\mathrm{D})/\mathrm{d}(\log M)]$$

which rearranged yields

$$\log{(M_1/M_2)} = 4R_s(V_0/V_\mathrm{p} + 1)(N_\mathrm{max})^{-1/2}$$
$$\cdot 1/[-\mathrm{d}(K_\mathrm{D})/\mathrm{d}(\log M)] \qquad (2)$$

REFERENCES

1 L. Hagel, in P. Dubin (Editor), *Aqueous Size-Exclusion Chromatography*, Elsevier, Amsterdam, 1988, Ch. 5.
2 W.W. Yau, J.J. Kirkland and D.D. Bly, *Modern Size-Exclusion Liquid Chromatography*, Wiley, New York, 1979, p. 116.
3 J.J. Kirkland, *J. Chromatogr.*, 125 (1976) 231–250.
4 T. Andersson, Pharmacia LKB Biotechnology, personal communication, 1992.
5 L. Hagel, in J.-C. Janson and L. Rydén (Editors), *Protein Purification, Methods for High Resolution Protein Separation and Analysis*, VCH, Deerfield Beach, FL, 1989, Ch. 3.
6 L. Hagel, *J. Chromatogr.*, 591 (1992) 47–54.
7 L. Andersson, *J. Chromatogr.*, 325 (1985) 37.
8 K. Nilsson and A.-C. Johansson, Kabi-Pharmacia, personal communication, 1992.
9 L. Hagel and T. Andersson, presented at the *11th International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Washington DC, October 20–23, 1991*, paper 214.
10 R.P. Frigon, J.K. Leypoldt, S. Uyeji and L.W. Hendersson, *Anal. Chem.*, 55 (1983) 1349.
11 P.L. Dubin and J.M. Principi, *Macromolecules*, 22 (1989) 1891.
12 M. Potschka, *Anal. Biochem.*, 162 (1987) 47.
13 W.W. Yau, J.J. Kirkland and D.D. Bly, *Modern Size-Exclusion Liquid Chromatography*, Wiley, New York, 1979, p. 297.
14 L.H. Tung, *J. Appl. Polym. Sci.*, 10 (1966) 1271.
15 E.M. Sörvik, *J. Appl. Polym. Sci.*, 21 (1977) 2769.
16 W.W. Yau, J.J. Kirkland and D.D. Bly, *Modern Size-Exclusion Liquid Chromatography*, Wiley, New York, 1979, p. 106.
17 G. Nilsson and K. Nilsson, *J. Chromatogr.*, 101 (1974) 137.