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Separation of serum proteins by high-performance gel-permeation column systems

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ABSTRACT

Combined TSK-Gel G4000SW–G3000SW and Zorbax GF450–GF250 columns and a Superose 6 column were evaluated for the analytical gel permeation chromatographic separation of serum proteins Serum elution profiles showed four distinct peaks, which were attributed to immunoglobulin M (IgM), α_2-macroglobulin, IgG and albumin A reproducible shoulder on the IgG peak could be attributed to IgA These purified serum proteins and other commercially obtained proteins with relative molecular masses between 1000 kDa and 10 kDa were chromatographed on the combined systems and the separate columns The frictional coefficient-based hydrodynamic radii of these proteins showed a linear relationship with the inverse error function complement of their partition coefficients Using this relationship, theoretical summation plots of the data obtained from the separate columns of a system correspond to plots that were calculated by treating the combined systems as one column The best resolution was obtained with the TSK columns, but all three column systems were suitable for the separation of clinically important immunoglobulins in serum The retention of positively charged proteins by the TSK columns becomes noticeable after relatively short usage and is a first sign of progressive loss of resolution

INTRODUCTION

It has recently been shown that high-performance gel permeation chromatography (HP-GPC) is well suited to the analytical separation of immunoglobulins from serum proteins [1,2]. HP-GPC can, therefore, be used for the detection and characterization of monoclonal immunoglobulin components in serum and for the demonstration of other dysproteinemias. HP-GPC is also used for the purification of monoclonal antibodies from ascites and other biologically active proteins from cell culture supernatants containing fetal calf serum [3,4]. In addition,

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HP-GPC is used for the quality control of immunoglobulins for therapeutic use [5] and serological procedures [6,7].

Gel permeation can be considered to be a transport process, and it is generally accepted that the partition (or distribution) coefficient of a molecule [K_D = (solute elution volume − column interstitial or void volume)/internal or pore volume of the column], depends on its effective size. Molecules, in this case native proteins, can, therefore, be treated as hydrodynamic particles of which the dimensions are a function of mass, shape and solvation [8]. The hydrodynamic particle can in turn be treated as an equivalent hydrodynamic sphere, and the equivalent radius can be related to hydrodynamic radii obtained from other transport properties, *e.g.* sedimentation, diffusion and viscosity [8,9] It has been shown that, for native globular proteins, the frictional coefficient-based as well as the intrinsic viscosity-based hydrodynamic radii represent the proteins' effective partitioning radii in gel permeation [10].

In this study we calibrated columns separately and as combined systems with proteins of known diffusion constants (D) Their hydrodynamic radii (R), calculated from the Stokes–Einstein equation, $R = kT/6\pi\eta D$, in which k is the Boltzmann constant, T the absolute temperature and η the viscosity of the medium, were related to the inverse error function complement of K_D, $R = A + B\,\text{inv.erfc}\,(K_D)$, according to Ackers [11]. This model assumes that the sizes of the pores within a gel permeation support follow a normal distribution A column can therefore be simply characterized by two empirical constants, A and B, in which A represents the maximum value of the pore size distribution and B the standard deviation of this value. As shown by the formula, a linear relationship between R and K_D exists. Our earlier studies [12] and those of Cabré and Canela [13] have shown that the best linear relationship is obtained by this model, compared with functions of the molecular mass *versus* functions of the elution volumes of calibrating proteins.

Most serum proteins have M_r values between 10^6 and 10^4 Da. For analytical separation within this range, and especially for separation of the clinically important immunoglobulins IgG (150 kDa), IgA (160 kDa) and IgM (900 kDa), we evaluated combined TSK-Gel G4000SW–G3000SW and Zorbax GF450–GF250 column systems. The Superose 6 column was used as a control. Using Ackers' model, hydrodynamic radii of the principal components of distinct fractions in the serum elution profiles were related to linear calibration plots of the combined systems and the Superose column. These columns may, therefore, be used to detect and quantitate immunoglobulins in serum. In addition, total serum may be used for calibrating gel permeation columns.

During this investigation we observed that retention of basic proteins is a first sign of deterioration of the TSK columns. This ion-exchange effect considerably decreases the resolution between immunoglobulins and serum albumin.

EXPERIMENTAL

Chemicals

Table I lists the proteins and their hydrodynamic parameters used in this study. Most references for diffusion constants were obtained from *The Handbook of Biochemistry and Molecular Biology* [14] Parameters for other serum proteins were obtained from *Wissenschaftliche Tabellen Geigy* [15]. R values were calculated according to the Stokes–Einstein equation or, if D values could not be traced, from the equation:

$$R = (f/f_0)(3VM_r/4\pi N)^{1/3} \quad (1)$$

in which V represents the partial specific volume, which has an average value of 0.74 ml/g for native proteins. For the frictional coefficient (f/f_0) of globular proteins an average value of 1.3 was used. The proteins 4, 6, 8, 9, 11, 13, 15 and 16 were purchased from Sigma, 5 from Behringwerke, 10 from Boehringer Mannheim and 12 and 14 from Serva. Human polyclonal pentameric IgM was purified

TABLE I

DIFFUSION CONSTANTS AND STOKES RADII OF NATIVE PROTEINS

Protein	$D \cdot 10^7$ [a]	R [b]	References
1 Human immunoglobulin IgM (pentameric)	1 73	123.9	Metzger (1970) [14]
2 Human α_2-macroglobulin	2 41	88 9	Schultze and Heremans (1966) [14]
3 Human immunoglobulin IgA (monomeric)	3.30	65.0	Heremans (1974) [14]
4 Human immunoglobulin IgG	4 00	53 6	Hall and Abraham (1984) [16]
5 Human serum albumin	6 10	35 1	Schultze and Heremans (1966) [14]
6 Bovine thyroglobulin	2 49	86.1	Edelhoch (1960) [17]
7 Bovine α-crystallin	2 55	84 1	Siezen and Berger (1978) [18]
8 Bovine γ-globulin	4 10	52 3	Butler (1983) [19]
9 Bovine serum albumin	5 90	36 3	Phelps and Putnam (1960) [14]
10 Bovine lactoperoxidase	5 95	36 0	Theorell and Pedersen (1948) [14]
11 Bovine α-chymotrypsinogen	9 48	22 9	Wilcox *et al.* (1957) [14]
12 Horse ferritin	3 21	66 2	Haen (1987) [20]
13 Horse myoglobin	11 30	19 0	Theorell and Pedersen (1948) [14]
14 Horse cytochrome c	13 00	16.5	Edsall (1953) [14]
15 Rabbit aldolase	4 63	46 3	Taylor and Lowry (1956) [14]
16 Horse-radish peroxidase	7.05	30 4	Cecil and Ogston (1950) [14]

[a] Diffusion constants in water at 20°C

[b] Radii in water at 20°C (η = 1 002 cP)

from pooled serum of 100 healthy blood donors (Dutch Red Cross Bloodbank) by euglobulin precipitation and subsequent chromatography on Superose 6B (Pharmacia) [21]. Human α_2-macroglobulin was purified from the serum pool by chromatography on Sepharose Blue (Pharmacia) [22]. Human polyclonal monomeric IgA was isolated from this same serum pool by the zinc sulphate method [23], affinity chromatography on Jacelin (Pierce) [24] and subsequent chromatography on Superose 6B. Human polyclonal IgG was purified by Protein A Sepharose chromatography (Pharmacia) and human albumin by Sepharose Blue chromatography. Except for the absence of dimers and trimers, the latter two proteins showed an elution behaviour identical with that of the purchased proteins. α-Crystallin from calf lenses was isolated by GPC as described previously [25]. Isolated proteins were analysed by sodium dodecyl sulphate electrophoresis and by their elution behaviour on HP-GPC columns

Chromatography

The TSK-Gel PWH guard column and the silica-based G4000SW and G3000SW columns (300 mm × 7 5 mm I.D.), manufactured by Toyo Soda, were purchased form Chrompack International (Middelburg, The Netherlands) The Zorbax Bioseries GF450 and GF250 columns, also silica-based, were purchased from Du Pont De Nemours (Boston, MA, U.S.A.). The Superose 6 column, consisting of highly cross-linked Sepharose spheres, was a gift from Pharmacia (Uppsala, Sweden). For the TSK and Zorbax columns we employed the guard column. When combined columns were used we connected them in sequence of descending pore size. For the TSK and Superose columns we used 0.15 *M* sodium chloride and 0.02 *M* sodium phosphate (pH 6.8) as elution buffer with flow-rates of 0.6 and 0.4 ml/min, respectively (LKB Model 2150 HPLC pump). For the Zorbax columns a 0.1 *M* sodium sulphate and 0.02 *M* sodium phosphate buffer (pH 6.8) at 0.4 ml/min was initially used, but increasing the sulphate concentration to 0.2 *M* and the phosphate concentration to 0.04 *M* gave a better separation of total serum without changing the elution volumes of the distinct peaks (see Results). For the viscosities (η) of water, the sodium chloride and the sodium sulphate buffers at 20°C, 1.002, 1 027 and 1.104 cP, respectively, were used. Valco stainless-steel tubing and connectors were used throughout the high-performance liquid chromatographic (HPLC) system (Chrompack). The Superose column was connected with Pharmacia SRTC-2 tubing connectors. Protein samples (100 μl), usually containing two or three different proteins at a concentration of *ca.* 3 mg/ml for each protein, were loaded onto the columns by a Gilson 231-401 autosampler or manually with a Valco loop injector. The proteins lactoperoxidase, myoglobin, phosphorylase, aldolase and horse radish peroxidase were not chromatographed on the Superose column. Detection of proteins was done by differential refractive index measurement (DRI, Melz Model LCD 201). Elution volumes of distinct protein peaks were calculated from the recordings (Hitachi 561 recorder; chart speed 5 mm/min) and double-checked by computer data anal-

ysis (Hewlett-Packard 3353 LAB DATA system). The total volumes of the columns were obtained by injecting elution buffer or L-lysine (Sigma). The void volumes were calculated from low-angle laser light scattering recordings (Chromatics KMX 6) by the appearance of high-molecular mass particles in serum, as described in an earlier paper [2]. This was in accordance with the void volume obtained from high-molecular-mass impurities in a solution of commercially obtained bovine fibrinogen (Sigma).

For column parameter estimates (A and B), regression analyses, and plots of R *versus* inv.erfc (K_D), SAS system programs (SAS Institute) were used. The root-mean-square error (RMSE) was calculated according to the equation.

$$\mathrm{RMSE} = [\Sigma(R - R_{\mathrm{pred}})^2/(n-2)]^{1/2} \quad (2)$$

in which R_{pred} represents the predicted R value. Theoretical summation plots of the normal distributions of both columns of a system were calculated according to the equation:

$$K'_{D3}(R) = \gamma_1 K'_{D1}(R) + \gamma_2 K'_{D2}(R) \quad (3)$$

for hypothetical proteins with R between 0 and 125 Å. K'_{D1} and K'_{D2} are the theoretical distribution coefficients for these radii defined by the parameters A and B of both columns of a system. γ_1 and γ_2 represent the internal volume (V_i = total column volume − void volume) fractions of each column of a system defined by: $V_{i1}/(V_{i1} + V_{i2})$ and $V_{i2}/(V_{i1} + V_{i2})$.

Deterioration of the resolution of the TSK columns was unmistakeable after two to three months of intensive use (*ca.* 250–300 injections for each column) and around this time the columns were re-evaluated.

RESULTS

Fig. 1 illustrates the elution behaviour of the purified serum proteins. It can be seen that the immunoglobulins IgG, IgA (mono- and dimer) and IgM have eluted separately. Fig. 2 shows the separation of pooled total serum by different column systems. A better resolution is obtained with the Zorbax columns by increasing the ionic strength of the elution buffer. The distinct peaks are numbered and their elution behaviour corresponds to that of the purified human serum proteins, as listed in Table I and shown in Fig. 1. Serum proteins with molecular masses below 60 kDa, which constitute 10% of total protein content of serum, are not resolved from the albumin fraction by any of the column systems.

Table II summarizes our experimental data and the exclusion limits as given by the manufacturer. The M_r values of most of the proteins used in this study are within the fractionation ranges of the systems. IgM (900 kDa) [14] and cytochrome c (12 kDa) [13] are thus the outer fractionation limits for the systems The

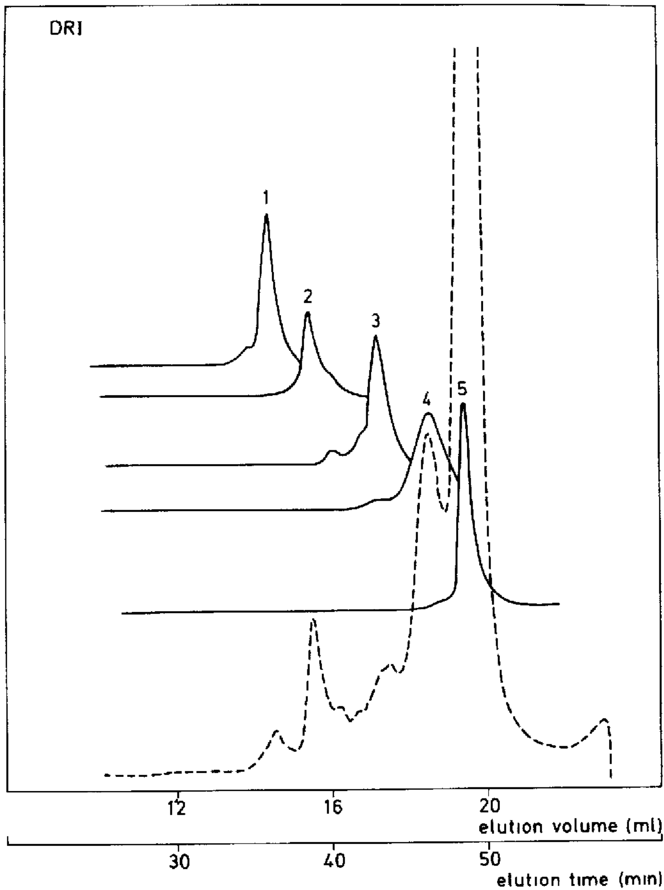

Fig 1 Elution profiles of purified serum proteins and total serum by Zorbax GF450–GF250 combination. Numbers correspond to the proteins in Table I

mean values of the pore size distributions (A) are small compared with the value of the deviation (B). A clearcut linear relationship of R *versus* inv.erfc(K_D) of the calibration proteins was observed for the individual columns and combined systems. The Superose column shows the best correlation and smallest error in the range tested. The highest resolution for the separation of total serum and especially for the separation of immunoglobulins from albumin is obtained by the TSK columns (Fig. 1 and Table II). However, during two to three months of extensive use of these columns (*ca.* 250–300 injections for each column), the resolution between these proteins decreases, owing to the retention of the positively charged immunoglobulins. In serum this results in the loss of resolution between the IgG and the albumin fractions, although the resolution of negatively charged proteins is initially not impaired Human IgG, chymotrypsinogen, myoglobin, aldolase and horse radish peroxidase, with isoelectric points above pH 6.8, *i.e.* pH 6–9 [26], 9.0 [27], 7.5 [28], 8.6 [29] and 9.0 [29], respectively, were

retained by *ca* 5% of their initial elution volumes on these columns. In addition, the base of each immunoglobulin peak broadens considerably. From this stage onward a progressive loss of resolution was observed, which resulted in an unacceptable loss of reproducibility.

Fig. 3 shows the computer-calculated plots of R *versus* inv erfc(K_D) for the different columns and column systems. The combined columns are treated as one column with one normal distribution. The dashed non-linear line for the column systems represents the theoretical summation of the empirically obtained normal distributions of the separate columns. It can be seen for the TSK system that the directly obtained and the summation plots diverge above R values of *ca.* 65 Å, which correspond to calculated M_r values above 425 kDa for globular proteins with a frictional coefficient of 1.3 For the Zorbax system these deviations occur above 85 Å and under 35 Å, which correspond to M_r values of 950 and 67 kDa, respectively. Within the limits of 10–65 and 35–85 Å for the TSK and Zorbax systems, respectively, considerable coincidence is obtained. For the total ranges the error between theoretical summation of empirically obtained distributions of the separate columns and treating the systems as one column is of similar magnitude (Table II, Fig. 3). The higher values of the RMSE for the combined systems compared with the Superose column can be attributed to deviations from the

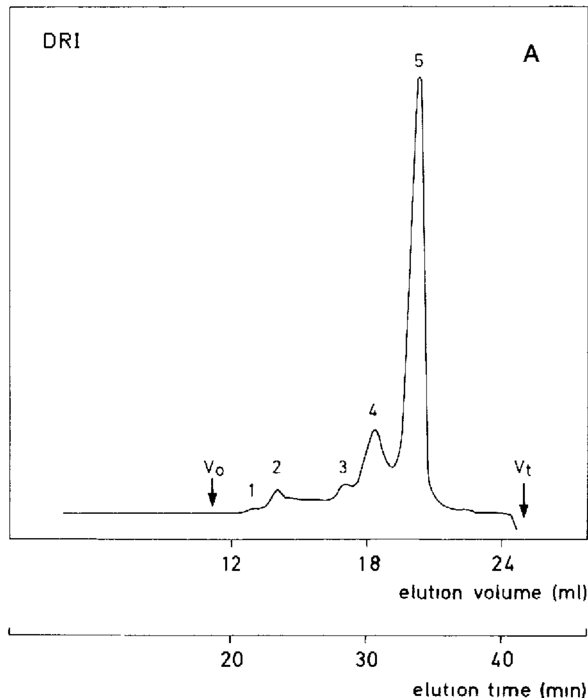

Fig 2

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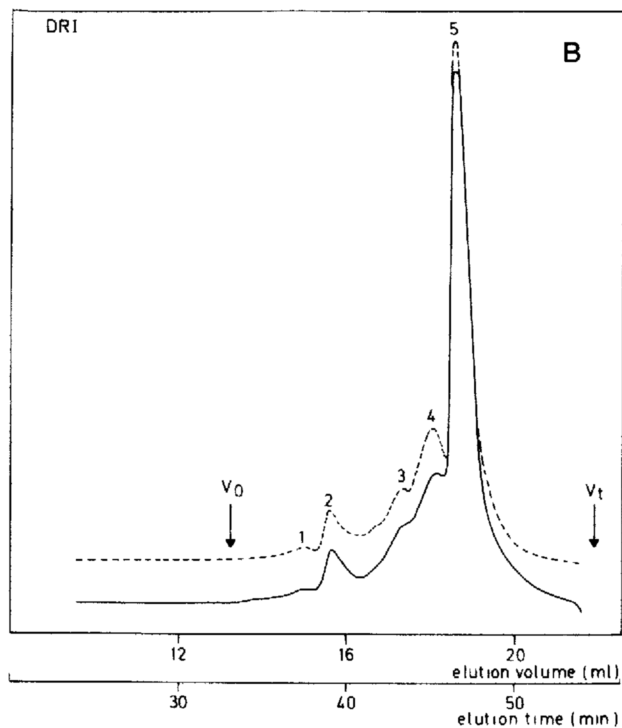


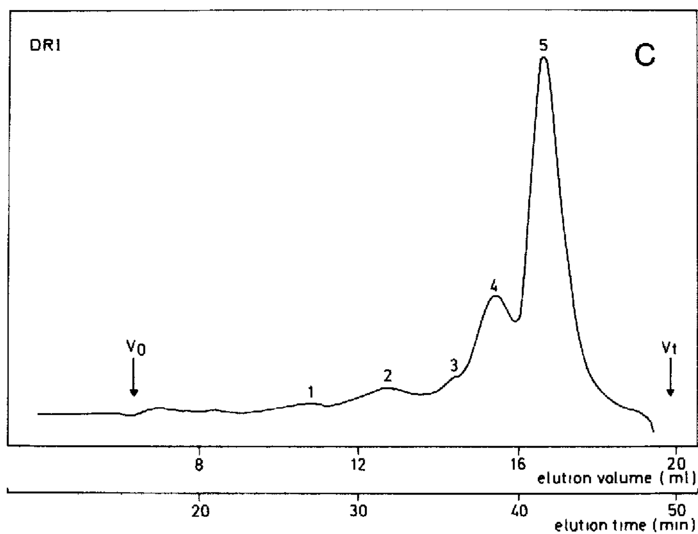
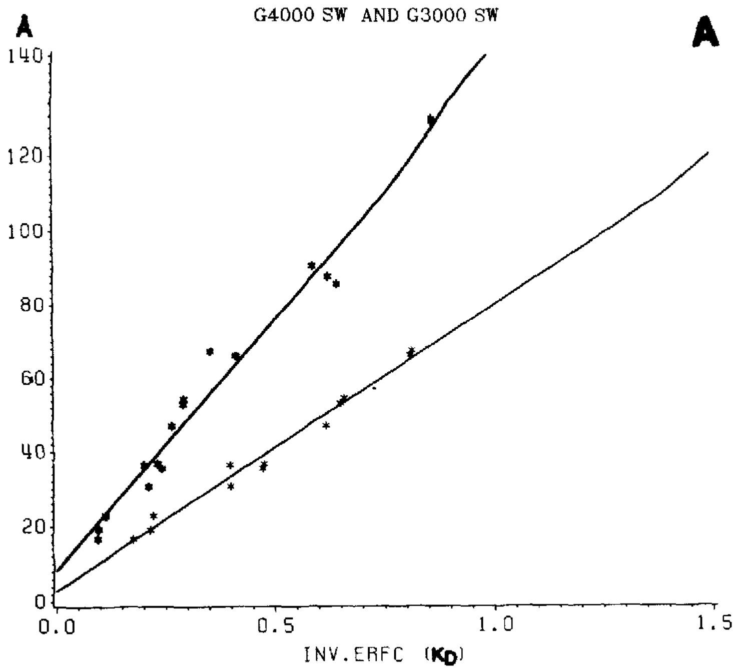


Fig. 2. Elution profiles of total serum (A) TSK-Gel G4000SW–G3000SW combination, (B) Zorbax GF450–GF250 combination (---, 0 24 *M* buffer; —, 0.12 *M* buffer), (C) Superose 6 Numbers correspond to the proteins in Table I



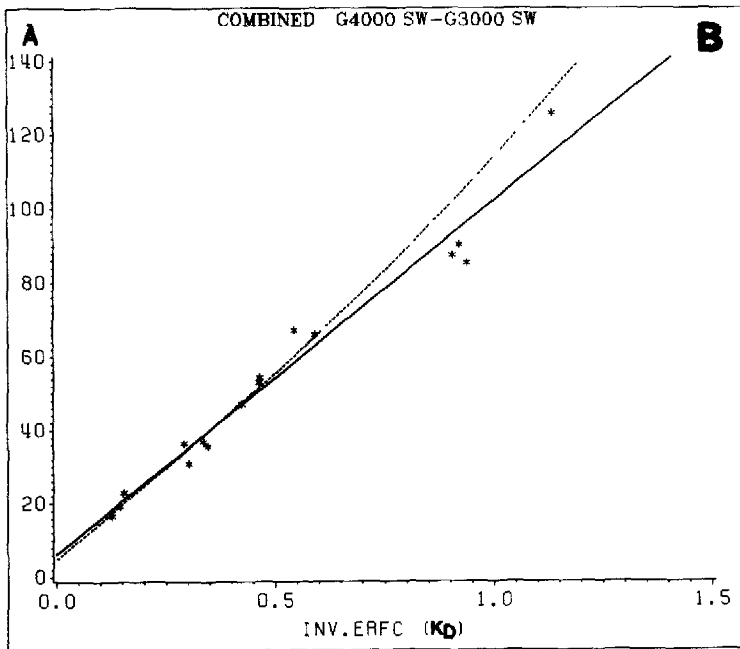


Fig 3

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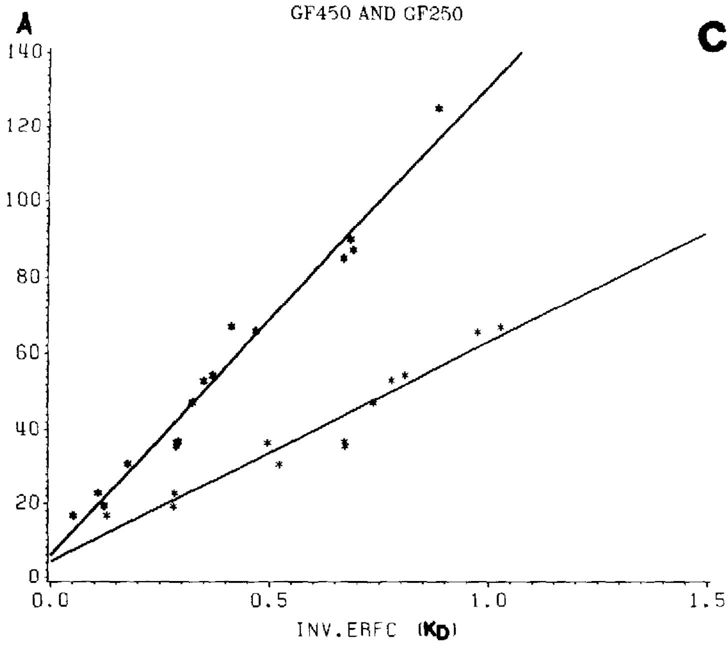

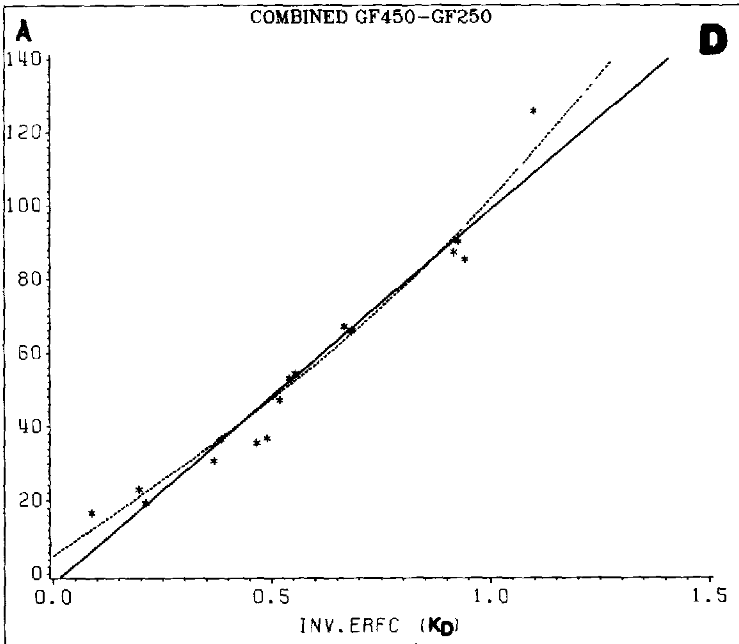

Fig 3

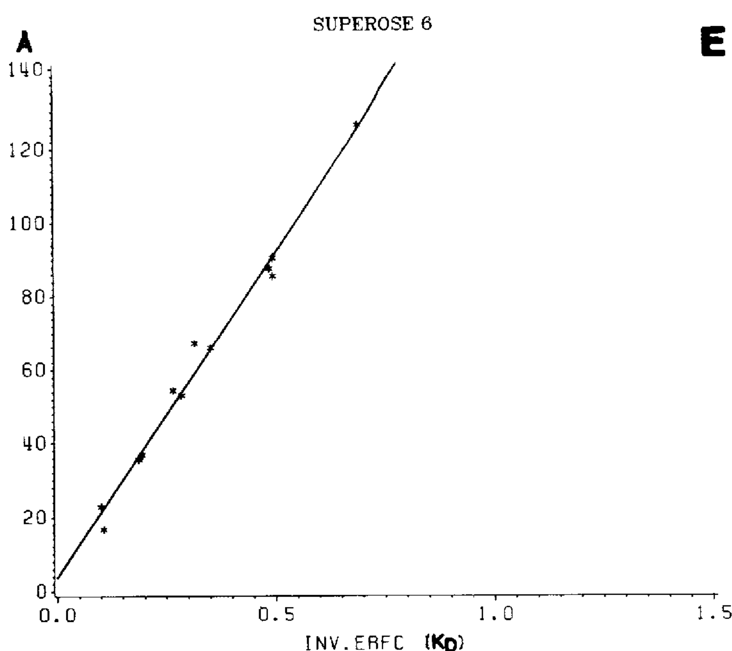

Fig 3 Plots of hydrodynamic radii *versus* inverse error function complement of partition coefficients and summation plots (A) (—) TSK G4000SW; (—) TSK G3000SW, (B) (—) TSK G4000SW–G3000SW system; (---) TSK summation, (C) (—) Zorbax GF450, (—) Zorbax GF250, (D) (—) Zorbax GF450–GF250 system, (---) Zorbax summation; (E) Superose 6

TABLE II

COLUMN CHARACTERISTICS

Column	Factionation range[a] (Da 10^{-3})	A (Å)	B (Å)	Correlation coefficient	RMSE[b] (Å)	Resolution[c]	
						6 and 9	8 and 9
G4000SW	1000–5	8 3	134 7	0 984	4 5	2 46	0.36
G3000SW	300–1	2 9	76 6	0 991	2 4		0.86
G4000SW–G3000SW		6 3	95 8	0 984	4 9/7 0[d]	3 90/3 32[e]	0.97/0 21[e]
GF450	900–25	6 0	126 4	0 986	5 1	2 68	0.36
GF250	250–10	4.3	59 0	0 966	4 7		0 47
GF450-GF250		−2 8	101 4	0 974	6 8/5 6[d]	3 44	0.51
Superose 6	5000–5	3.7	175.6	0.993	2.8	2.07	0.67

[a] Exclusion limits from instruction manual

[b] Root-mean-square error according to Eqn 2

[c] Resolution of proteins thyroglobulin (6) or γ-globulin (8) and albumin (9) according to the difference in elution volumes (ΔV) divided by the mean peak width of these proteins, $(W_6 + W_9)/2$ or $(W_8 + W_9)/2$, in ml/ml

[d] RMSE for summation

[e] Resolution after 250–300 injections

regression line in the outer fractionation limits of the columns. This can be seen for IgM, which deviates considerably from the regression lines of the combined columns. Its hydrodynamic volume, however, appears to be correct as judged by the data from the separate columns. The immunoglobulins IgA and IgG elute according to their hydrodynamic volume on all columns. Correcting R values for different buffer viscosities does not change the statistical outcome (differences for RMSE $<$ 0.5%).

DISCUSSION

Our results show that purified immunoglobulins, α_2-macroglobulin and albumin are separated according to their hydrodynamic volume and represent distinct peaks in the serum elution profiles. Electrophoretic analyses [30,31] and nephelometric quantitation of immunoglobulins G, A and M (P Faaber and W. Flapper, unpublished results) in HP-GPC fractions of total serum confirm that the elution volumes of these proteins do not change in the presence of other serum proteins. In an earlier paper we compared protein concentrations, calculated from peak areas in the elution profiles of total serum, with protein concentrations of electrophoretic fractions [2]. From these results we conclude that the human serum proteins considered constitute a considerable portion of the peaks in the elution profiles. These distinct peaks with well defined hydrodynamic volumes can serve as a reference when dealing with other serum components or pathological immunoglobulin fractions.

Combining the columns not only increases the effective fractionation range for complex protein samples such as serum, which contains more than 50 components, but also increases the resolution between proteins with smaller differences in M_r (*e.g.* IgG and albumin, Table II). Therefore, IgM is separated from α_2-macroglobulin and IgA from IgG (Figs. 1 and 2). About half of the serum proteins have M_r values between 80 and 35 kDa, and consequently R values between *ca.* 40 and 30 Å (*e.g.* transferrin: M_r = 77 kDa, R = 39 Å, and β_2-glycoprotein I: M_r = 40 kDa, R = 30 Å), and amount to only 5% of the total protein concentration in serum [15]. Hence, these proteins are not separated by these HP-GPC columns from the albumin fraction, which constitutes 50% of the serum protein content.

Combining the columns can lead to non-linear calibration plots and artificial peaks [32]. In our studies no additional peaks were observed in the elution profiles of the systems, and there was no anomalous elution behaviour of proteins. This was confirmed by the Superose data. Deviations from the calibration lines do, however, increase in the outer fractionation ranges of the systems. This can be attributed to the non-overlap of the ranges of the columns of a system. Within defined fractionation limits (see Results), the TSK and Zorbax column systems can be treated as one column using Ackers' linear relationship. The summation of the broad pore size distributions of the columns of a system produces, therefore, a

distribution that can be characterized as an almost normal one. This considerably simplifies the calibration procedure of these column systems.

The hydrodynamic volume of proteins is ultimately limited by their M_r values and methods that use derivatives of the M_r to calibrate columns are applicable by virtue of this fact. The limitations of the linear relationship of log M_r *versus* elution volume for the calibration of columns become more apparent when columns with higher resolutions are used [33]. Errors in M_r, *e.g.* too low for IgG [2] or too high for ferritin [34], are due to the differences in shape and solvation of these proteins compared with other calibrating proteins. Assigning an effective hydrodynamic volume to a protein and relating this to some derived magnitude of the elution volume produces better results. Indeed, it has been shown that pore size distributions of gel permeation columns can be defined as polynomes, which are calculated directly by relating the K_D of a number of proteins to their hydrodynamic volumes [35]. Limitations of this method are the non-linearity of the calibration plots and non-ideal elution behaviour of proteins caused by hydrophobicity or asymmetry [36], which produces errors in the pore size distributions. Ackers' relationship is less sensitive to these errors because it leads to an averaged pore size distribution.

The ion-exchange effects of silica supports at low ionic strengths [37,38] can be used advantageously for the separation of complex protein mixtures, *e.g.* lens crystallins [39] A serious drawback of the TSK columns is the non-ideal elution behaviour of proteins caused by ion-exchange effects at physiological ionic strengths, which is noticeable after relatively short usage. In effect, positively charged proteins are retarded on and negatively proteins are excluded from the columns [37]. Human and bovine IgG also show an increased dispersion, which presumably reflects the charge heterogeneity in these populations. These phenomena are a first sign of the progressive deterioration of resolution and can be attributed to the loss of the bonded phase and the exposure of silanol groups, which eventually results in solvation of the silica matrix [40]. In our laboratory the performance of the Zorbax columns remained constant during two years of almost daily use. The zirconium oxide coating of the spheres of these columns is responsible for their long lives [41]. The great stability of Superose supports has been amply dealt with [42].

We have shown that TSK and Zorbax systems and the Superose column are well suited for the separation of immunoglobulins from other serum proteins Frictional coefficient-based hydrodynamic radii of these proteins, which elute in the distinct fractions of the serum elution profiles, can be related to linear calibration plots using Ackers' relationship to characterize gel permeation supports. The separation of immunoglobulins from most other serum proteins afforded the possibility of analysing disturbances in these proteins. This paper will serve as a reference when dealing with abnormal immunoglobulin fractions in serum, which will be investigated in a forthcoming paper

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