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Analytical gel filtration of dextran for study of the glomerular barrier function

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ABSTRACT

Analytical gel filtration was used for the study of molecular size distribution of clinical dextran in serum and urine for the purpose of evaluations of changes in the human glomerular barrier function. The column was calibrated in terms of solute size using a simple and accurate technique recently described. Only one sample of a dextran possessing a broad molecular mass distribution was necessary for the calibration procedure and the calculations were performed using an ordinary spreadsheet. The accuracy of the calibration, as evaluated by protein samples, is better than 95%. The simplicity makes the method suitable for use in laboratories not normally specializing in analytical gel filtration. Calibration in terms of size is preferably done with respect to viscosity radius to obtain relevant information about the permeability of dextran into porous membranes.

INTRODUCTION

Characterization of molecular mass distributions of dextran with analytical gel filtration was first described by Granath and Flodin [1] approximately 30 years ago. Granath and Kvist [2] reported some application areas of analytical gel filtration, including the study of the human renal threshold, which they reported to be about 55 000 in mass-average molecular mass of dextran. The technique was soon used for the study of glomerular barrier function both in animals and in humans (see, *e.g.*, refs. 3 and 4). The method is of particular interest in cases of pathological proteinuria due to loss of glomerular sieving function, particularly in diabetic and glomerulonephritic proteinuria. The method evaluates neutral dextran sieving in contrast to negatively charged proteins in the clinical setting. Negatively charged particles such as proteins are more restricted than neutral dextran of similar size, probably because of the negative charges of fixed barrier proteins. With these limitations dextran clearance is still the most valid and clinically important method for *in vivo* estimation of glomerular sieving function [5–7].

One drawback of the method is the tedious calibration procedure, requiring the use of many samples and involving dedicated software for the calculations [8,9]. Also, the interpretation of the result into solute size will require some precautions, *i.e.*, different substance classes will show different relationships between size and molecular mass, and this may also vary within the substance class [10–12].

Recently, a procedure in which the column was calibrated through the use of a dextran of a broad and known molecular weight distribution was presented [13]. The simplicity of the method

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made it very apt for the use in laboratories with limited experience in analytical gel filtration and, furthermore, it requires no sophisticated software for the calibration and evaluations. We here report our experiences of using this procedure for evaluations of molecular size distributions of dextran in serum and urine to study the glomerular barrier function in humans.

CALIBRATION PRINCIPLE

Gel filtration, or aqueous size-exclusion chromatography, is frequently used for the determination of size or size distributions of solutes. In the absence of secondary interactions the solutes are eluted strictly according to decreasing size. The column may either be used simply as a separating device and the size of the eluted species determined on- or off-line, *e.g.* by light scattering, or it may be calibrated by the use of reference substance(s) of known size and the size is then related to the elution volume. For the detection of dextran in body fluids, the latter approach combined with a selective chemical assay of dextran content is necessary. Traditionally, the column is calibrated through the use of several standards for which an estimate of the molecular mass distribution, *e.g.*, mass-average molecular mass (M_w) or number-average molecular mass (M_n), has been determined and the calibration curve is obtained by an iterative procedure to yield the conventionally true values of the estimates [8]. This procedure requires dedicated software for the calculations. Also, in cases where the column is calibrated *versus* the peak values, software is needed for regression of a calibration curve and calculation of size estimates. It may also be noted that the limited number and spread of calibration points used for the regression will of course affect the accuracy of the calibration.

The procedure described by Hagel and Andersson [13] utilizes all the information embedded in the molecular mass distribution curve of a polymer, *i.e.*, the relationship between cumulative mass fraction and molar mass [14]. This relationship may be obtained by traditional methods such as light scattering of fractions after ethanol precipitation, or by using modern detection principles such as multi-angle laser-light scattering of column effluent. The information may also be obtained from gel filtration analysis using carefully calibrated columns, preferably employing a large number of calibration points.

The column to be used is calibrated over the size range of interest by gel filtrating a sample possessing a suitable size distribution, *i.e.*, there is no need for calibration of the entire separation range of the column. The concentration of solute is determined by chemical assay, either of collected fractions or by on-line analysis, or may be monitored by an on-line detector if no interfering substances are co-eluted. The concentration (expressed in units or peak height) at increasing elution volume is noted and the cumulative amount of substance computed. The molecular mass corresponding to the cumulative amount is then calculated from the known molecular mass distribution curve of the sample. Finally, the molecular mass is converted to solute size and, thus, the calibration curve relating solute size to elution volume is established. This procedure may be performed manually or by a personal computer using a simple spreadsheet.

The size-determining factor in gel filtration has yet to be confirmed [12,15–17]. However, the size of globular proteins, expressed either as Stokes radius or as viscosity radius (sometimes called hydrodynamic radius), is closely depicted by the viscosity radius of dextran [15,16]. On the other hand, proteins and dextran do not lie on a common calibration curve when Stokes radius is used as size parameter for dextran [15]. It may from these reports be concluded that in order to study effects based on the size of globular proteins the calibration of the gel filtration column should be made in terms of viscosity radius if dextran is used as test probe. This viscosity radius is calculated from [18]:

$$R_h = 0.271 \cdot M_r^{0.498} \quad (1)$$

and the Stokes radius is given by [7]:

$$R_{St} = 0.33 \cdot M_r^{0.463} \quad (2)$$

As can be seen from these equations, Stokes radius yields values 12–18% lower for solute size

than viscosity radius for solutes in the range 25–70 Å.

EXPERIMENTAL

Chromatography

The column, XK 16/100, was packed with Sephacryl S-300 SF to a bed height of 93 cm according to the manufacturer's instructions. The column packing was controlled visually by running a sample of Blue Dextran 2000. The column was connected to a MicroPerpex peristaltic pump (yielding a nominal flow-rate of 0.4 ml/min) and a fraction collector, RediFrac. All equipment was obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). The eluent was prepared by dissolving 6.9 g of N-tris(hydroxymethyl)methyl 2-aminoethanesulphonic acid, 99% (TES; Sigma), 105 g of sodium chloride (99.5%, Merck), and 3 g of trichlorobutanol (98–99%, Merck) in 2000 ml of distilled water, adding 0.1 ml of mercaptoethanol (98%, Aldrich), adjusting the pH to 7.0 and completing the volume to 3000 ml. A 1-ml aliquot of untreated urine or serum was applied to the column, and up to 35 fractions over the fractionation range of interest were collected and subjected to chemical assay of dextran content. The fraction size, approximately 2 g, was determined by weighing.

Chemical assay

A detection principle that discriminates between dextran and high-molecular-mass constituents in body fluids was needed for this study, *i.e.* a general detection principle such as refractive index is not applicable. The chemical assay proposed by Scott and Melvin [19] has frequently been used for analysis of dextran. With this method dextran is hydrolysed to glucose and converted to 5-hydroxymethylfurfural when heated with strong acid. Furfural condenses with anthranol to a blue–green chromophore showing an absorption maximum at 625 nm. Thus, all glucose-containing solutes will yield a positive response. It may also be noted that some types of preservatives, *e.g.* sodium azide, are not compatible with the anthrone method. However, for our purpose the anthrone method is very suitable. The concentration of dextran in the fractions was determined by mixing 1 ml of sample with 2 ml of anthrone reagent (0.5 g of anthrone in 250 ml of sulphuric acid) and immersing the mixture in a boiling water bath for 7 min [19]. The absorbance at 625 nm, using 2 ml of reagent plus 1 ml of buffer treated as above as reference, was taken as the dextran concentration. Fractions displaying an absorbance exceeding 1 were diluted and re-evaluated.

Calibration

The column was calibrated using a dextran sample possessing a broad molecular mass distribution, *i.e.* with a mass average of 72 300 and a polydispersity, M_w/M_n, of 2.4, which had been determined by size-exclusion chromatography [20]. This dextran yields an accurate calibration range, corresponding to the 5th and 95th percentiles of the distribution, from 190 000 to 10 000 in molecular mass, which equals 115–27 Å in solute size of dextran [14]. Information from multi-angle laser-light scattering (MALLS) was used to judge the accuracy of the molecular mass distribution of the calibration sample [21]. A 1-ml aliquot of the calibration dextran, containing 3 mg, was applied to the column, and 1- or 2-ml fractions were collected in the range of interest (*i.e.* approximately between 80 and 150 ml). The content of dextran in the fractions was determined by the anthrone method, as described above, and the cumulative area as a function of elution volume was calculated. The calibration curve was obtained by calculation of the molecular mass for which the integral mass coincides with the cumulative area of each fraction [14]. The calibration in molecular mass was then converted to viscosity radius through the use of eqn. 1 or to Stokes radius through the use of eqn. 2. Calculations and reports were performed using a dedicated application of Excel 3.0 (from ref. 22). The accuracy of the calibration was tested by chromatography of two proteins of known size, *i.e.* ferritin and bovine serum albumin. The fractions were in this case analysed for absorbance at 280 nm, and the exact maximum was determined from a third-degree polynomial fit to the five concentrations surrounding the maximum.

Evaluation of size distributions

The distribution of dextran in urine as compared with that of plasma was studied by applying 1 ml of untreated sample on the column, collecting 2-ml fractions and analysing these for dextran content with the anthrone method. The absorbance values were entered into a spreadsheet (SECSoft [22]), which calculated elution profiles for the samples and the ratio of the dextran content of urine to that of serum as a function of solute size.

RESULTS AND DISCUSSION

Calibration of the column

A typical elution profile of the calibration substance is shown in Fig. 1. The fractionation and subsequent manual analysis yields a variability in the determination of dextran content. Fortunately, this variability does not affect the calibration curve established, since smoothing of the raw data, using a three-point moving average, prior to calculations had no noticeable effect (*i.e.* less than 0.3%) on the final result. The calibration curve obtained from the integral method is shown in Fig. 2. Owing to the large number of data points, *i.e.* fractions, there is no need to apply any curve-fitting procedure to achieve a smooth curve. The figure also illustrates the reliability of the molecular mass distribution data of the reference sample as determined by gel filtration on a carefully calibrated column set. By using data for the molecular mass distribution of the reference sample, as obtained by an absolute method, *i.e.* on-line MALLS employing a different column set [21], more or less identical calibration curves were obtained. The accuracy of the method, as estimated from the viscosity radii of dextran and proteins, is good, *i.e.* better than 5%, as shown by the data in Table I. Using Stokes radius as size estimate will yield too low an estimate of protein size. Unfortunately, literature data for the viscosity radius of ferritin differ considerably. The reproducibility of the calibration procedure as evaluated by running the sample three times showed a range of roughly 1% in the middle of the calibration area, which increases slightly at the extremes of the calibrated domain (Table II). This is because of variations in the selection of start and end points of sampling, which will influence the cumulative fractions and thus the calculated size. The influence in the centre of the calibration range, *i.e.* 70–30 Å, will be small. However, to obtain an accurate calibration of the entire range it is important that the sampling of fractions is made over the entire separation range of the column! It is also advisable to check

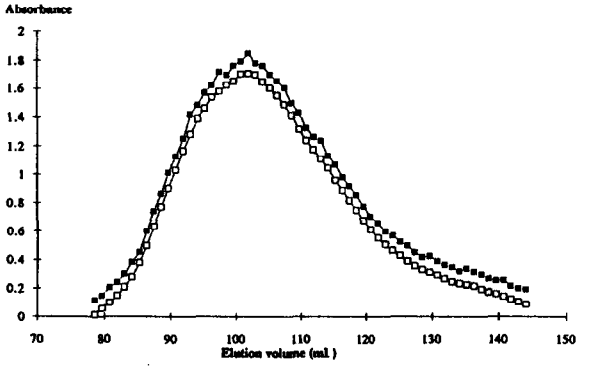

Fig. 1. Typical elution profile of the calibration substance. The sample, dextran with M_w = 72 300 was chromatographed on Sephacryl S-300 SF. The absorbance, as obtained from the anthrone analysis, is plotted *versus* the elution volume corresponding to the midpoint of each fraction. The smoothed curve was calculated using a three-point moving average (the curve is displaced −0.1 absorbance units to increase the readability). ■ = Original; □ = smoothed.

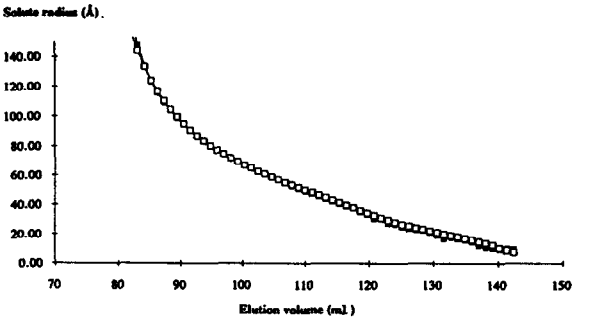

Fig. 2. Calibration curve of dextran on Sephacryl S-300 SF. The solute radius as a function of elution volume was calculated from the elution profile (*e.g.*, see Fig. 1) and the molecular mass distribution of the sample by employing the integral method and converting molecular mass to viscosity radius with the aid of eqn. 1. Calibration curves were calculated using data for molecular mass distribution of the reference sample as obtained by gel filtration (□) and light scattering (■).

TABLE I

EVALUATION OF SOLUTE SIZE FROM THE CALIBRATION CURVE

Column	Solute	Calculated size (Å)[a]		Nominal size (Å)[b]		Deviation (%)[c]		Ref.
		R_h	R_{St}	R_h	R_{St}	R_h	R_{St}	
911119	Dextran M_p 66 700	66.4	54.9	68.4	56.5	−3.0	−3.0	23
911128	Dextran M_p 66 700	69.6	57.4	68.4	56.5	+1.8	+1.8	23
920123	Bovine serum albumin	35.6	30.8	36		−1.1		12
					36.1		−14.7	15
920521	Ferritin	68.0	56.2	65.6	67.1	+3.6	−16.2	24
				61		+11.4		12
					59.3		−5.2	15
	Bovine serum albumin	37.4	32.2	36		+3.9		12
					36.1		−10.8	15
920903	Ferritin	66.6	55.1	65.6	67.1	+1.5	−17.9	24
				61		+9.1		12
					59.3		−7.1	15
	Bovine serum albumin	34.7	30.0	36		−3.6		12
					36.1		−16.9	15

[a] Size calculated from the calibration curve with aid of eqns. 1 and 2.
[b] Nominal size of dextran calculated from nominal molecular mass corresponding to the peak apex, M_p, (ref. 23), eqns. 1 and 2.
[c] Relative to nominal size.

the calibration curve with a number of samples covering the entire calibrated range.

The data in Table I illustrates that calibration of the column in terms of viscosity radius yields a more accurate estimate of protein size than calibration in terms of Stokes radius, which may yield too low an estimate of approximately 15–25% [15]. Unfortunately in many investigations dealing with study of barrier function, the molar mass of dextran has been converted to solute size in terms of Stokes radius, which, in view of recent research, turns out to be incorrect for the interpretation of size of globular proteins [15,16].

TABLE II

REPEATABILITY OF THE CALIBRATION PROCEDURE

Note that the valid calibration range is from 27 to 115 Å.

Retention volume (ml)	Calculated solute size (Å)			Range (Å)
	Run 1	Run 2	Run 3	
85	135.9	139.2	137.5	3.3
90	102.5	101.0	102.6	1.6
100	70.1	69.4	70.4	1.0
110	51.6	51.3	51.7	0.4
120	35.6	36.0	36.7	1.1
130	22.7	23.5	23.8	1.1

Evaluation of samples

The influence of the glomerular barrier function on the excretion of dextran of various size is illustrated in Fig. 3. The distribution of dextran in urine is displaced towards molecules of lower size owing to the restriction in the glomerular barrier, which increases with size. The peak eluting close to the total liquid volume of the column comes from inulin, a low-molecular-mass polyfructose (M_r 5200). Inulin clearance provides the measurement of glomerular filtration rate (GFR, ml/min), and is the standard used for measuring ultrafiltration of small solutes and water through the glomeruli. The clearance of a substance, C_x, is calculated from [25]:

$$C_x = U_x V / P_x \quad (3)$$

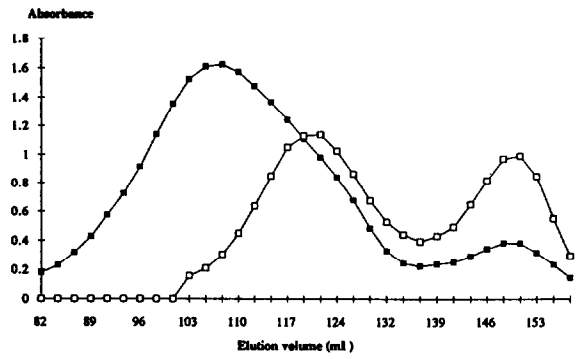

Fig. 3. Typical elution profiles of dextran in serum (■) and urine (□) samples. A 1-ml sample was run on the column, Sephacryl S-300 SF, and the collected fractions analysed with the anthrone method. The first peak represents dextran and the second peak represents inulin. All fractions displaying an absorbancy exceeding 1 were diluted and re-evaluated.

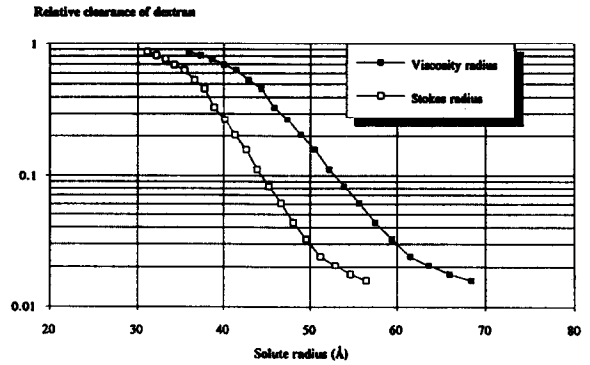

Fig. 4. Relative clearance of dextran, C_{dex}/C_{in}, as a function of solute size. Clearance was calculated from the elution profiles (*e.g.* see Fig. 3) and eqn. 4. Solute size, expressed as viscosity radius of dextran, was calculated from the calibration curve (Fig. 2). For the purpose of comparison with earlier published results, Stokes radius (eqn. 2) is also given. Please note that Stokes radius will yield too low an estimate of size of globular proteins (see text for explanations).

where U_x is the concentration of a substance (like inulin or dextran) in urine, V is the urine volume for a given period and P_x is the concentration of the substance in plasma. The clearance of dextran of different sizes can be calculated by the same formula, using measurements of fractions of concurrent dextran size in urine and blood. The ratio between dextran (dex) and inulin (in) clearance is calculated from:

$$C_{dex}/C_{in} = (U_{dex}V/P_{dex})/(U_{in}V/P_{in})$$
$$= (U_{dex}/P_{dex})/(U_{in}/P_{in}) \quad (4)$$

This ratio approaches 1 as the size of the dextrans becomes small and the molecules pass freely through the glomerular barrier like inulin. Greater restriction applies for dextrans of larger size, and the ratio falls towards zero when the dextrans are so large that they no longer appear in the urine. Thus the ratio is a meaningful tool in the evaluation of the relative restriction of large molecules compared with those freely filtered. The calculated clearance of inulin was no different whether inulin was measured in the urine and blood samples in presence or absence of dextran. This is in agreement with experience reported elsewhere [26].

The relative clearance of dextran as a function of solute size, expressed as viscosity radius of dextran, is shown in Fig. 4 (the corresponding plot for Stokes radius is also given, however, as stated above, we recommend that evaluations are made with respect to viscosity radius to yield an accurate estimation of equivalent size of globular proteins). In some experiments we noticed oscillations of clearance for small solutes. This problem was attributed to the influence of interfering inulin, therefore the data are truncated at a viscosity radius of 30 Å. To obtain data for smaller molecules, another marker of GFR that does not interfere with the anthrone reaction, for example ^{51}CrEDTA, is more appropriate than inulin. This will also avoid problems with examinations of diabetic patients in whom high blood and urine glucose levels may interfere with the dextran as well as the inulin measurements [27]. In order to check the repeatability of the evaluation step, the complete analysis was repeated five times. The relative error in the determination of dextran clearance is illustrated by Fig. 5. The large uncertainty for solutes of small size, *i.e.* smaller than 45 Å, is evident, however for solutes of primary interest in the evaluation of the glomerular barrier function, *i.e.* larger than 50 Å, the relative error is acceptable (*i.e.* 10–20%). Estimation of the relative dextran clearance also has its limitations for large molecules. The urine concentrations of dextran of sizes exceeding 70 Å in viscosity radius is so low that the extinctions

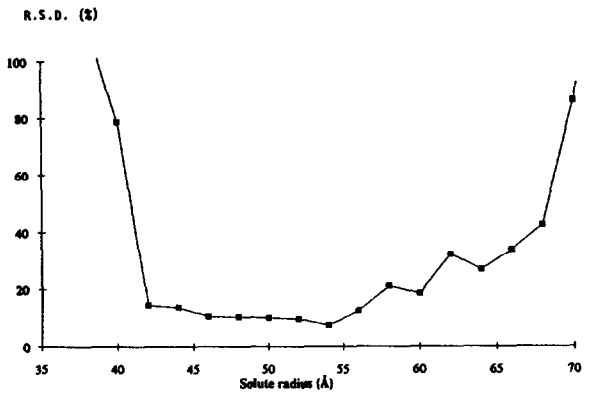

Fig. 5. Precision of the determination of relative clearance as calculated by repeating the entire chromatographic assay five times. The relative standard deviation was calculated for various solute viscosity radii.

with the anthrone method approaches background values, which makes data for clearance unreliable. However, as the clearance approaches zero, the variability is in absolute terms still very small.

One critical issue when it comes to interpretations of the size of dextran into size of proteins is the variability of size of random coils, *i.e.* dextran, as compared with compact charged ellipsoids, *i.e.* proteins, in different solvents. The use of dextran viscosity radius in dilute solutions as representative of protein size in body fluids will of course only be correct as long as the size of the solutes does not vary considerably with ionic strength. The intrinsic viscosity of native dextran was found to increase slightly with ionic strength (*i.e.* by 5% when going from water to 4 *M* sodium chloride), and the radius of gyration increased by 10% [28]. This was attributed to salt solution being a better solvent for dextran than pure water. The viscosity radius of dextran is rather insensitive to moderate ionic strength (*i.e.* up to at least 0.5 *M* [29]). The ionic strength of urine does not exceed that of plasma and the plasma sodium ion concentration is about 0.15 mol/l; the chloride ion concentration is about 0.1 mol/l and other anions account for 0.05 mol/l. Therefore, ionic strength probably has a minor impact on dextran size in this setting. For charged molecules, such as proteins, the situation is somewhat different. In solutions of high ionic strength the molecules are less stretched out because of screening of the charged surface. This is exemplified by the reduced intrinsic viscosity of serum albumin in salt solutions [30]. This illustrates the importance of selecting probes calibrated in the solvent to be studied.

The use of flexible molecules as a model for transport of compact proteins through membranes must also be addressed. The possibility of vermicular motion of flexible molecules may, in theory, enable then to penetrate also small pores, *i.e.* the molecules would then display an apparent smaller size than determined hydrodynamically. However, Davidson and Deen [31] found in a study of transport of flexible molecules through porous membranes that, owing to solvent permeability, random coil molecules appeared physically larger than an impermeable sphere of the same Stokes radius.

These observations support the use of dextran for study of membrane pore dimensions in aqueous solvents of moderate ionic strength and the expression of size of globular proteins in terms of viscosity radii of dextran.

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