Journal of Chromatography, 483 (1989) 406–412
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 008

Note

High-performance gel permeation chromatography of meningococcal polysaccharides

P. NEWLAND, B. BINGHAM, E. TARELLI and A. H. THOMAS*

Division of Chemistry, National Institute for Biological Standards and Control, Blanche Lane, Potters Bar, Herts. EN6 3QG (U.K.)

(Received July 3rd, 1989)

Meningococcal polysaccharide vaccines consist of one or more purified polysaccharides obtained from suitable strains of *Neisseria meningitidis* groups A, C, Y and W135. The polysaccharides are polymers of 1–6-linked N-acetylmannosamine phosphate (group A); 2–9-linked N-acetylneuraminic acid (group C); 2–6-linked 4-O-glucosyl-N-acetylneuraminic acid (group Y) and 2–6-linked 4-O-galactosyl-N-acetylneuraminic acid (W135), all have the α–D configuration and contain some O-acetyl groups. The polysaccharides must be safe and capable of inducing the production of satisfactory levels of specific antibody in man.

So far there is no animal model for the evaluation of the clinical potency of meningococcal polysaccharide vaccines and therefore various *in vitro* physico-chemical and serological tests are used in the laboratory as indicators of the vaccines' *in vivo* specificity and immunogenicity[1]. It has been demonstrated that the immunogenicity of polysaccharide antigens in man is directly related to their relative molecular mass and this can be determined in the laboratory by the distribution coefficient, K_D, using Sepharose gel permeation chromatography (GPC). The K_D value is inversely correlated with the relative molecular mass; the lower the K_D value the more immunogenic the antigen. The distribution coefficient may then be used as an indicator of the immunogenicity of polysaccharide vaccines. The group A polysaccharide is intrinsically unstable and readily depolymerizes at ambient temperature thus the K_D value may be used to monitor the stability of the vaccine. International requirements for meningococcal polysaccaride vaccines were introduced in 1976[2], the specification for molecular size was amended in 1977[3] and the test using gel permeation chromatography is still a pharmacopoeial requirement[4].

The present study was undertaken to determine if the recently introduced high-performance GPC columns would be suitable for the analysis of the meningococcal polysaccharides with the the advantages of a faster separation and a smaller sample requirement.

0021-9673/89/$03.50 © 1989 Elsevier Science Publishers B.V.

EXPERIMENTAL

Materials

Meningococcal polysaccharides, groups A, C, Y and W135 were kindly provided by Dr. M. Corbel, Division of Bacteriology, National Institute of Biological Standards and Control, U.K. Pullulans, non-branched linear polysaccharides used for the calibration of aqueous GPC columns were purchased from Showa Denko (Japan).

GPC

The pharmacopoeial method[4] for the determination of molecular size was followed using a column, 1000 mm × 16 mm, packed with Sepharose 4B CL and a mobile phase of 0.2 *M* ammonium acetate pH 7.0 ± 0.2 with a flow-rate of approximately 35 ml/h. Between 3 and 5 mg of polysaccharide in 1 ml of mobile phase were applied to the column and the eluate monitored with a differential refractometer (R403, Waters Assoc.), sensitivity $0.1 \cdot 10^{-3}$ ΔRI (RI = refractive index) maintained at 34°C and registered on a flat-bed recorder (Venture Servoscribe, Smiths). Fractions of 3 ml were collected and the polysaccharide content determined by the appropriate method. Group A polysaccharide was estimated by the amount of phosphorus[5] and group C, Y and W135 polysaccharides were estimated by the amount of sialic acid[6].

For high-performance GPC, samples (5 mg/ml) were prepared in the mobile phase and injected using a sample injection valve (Model 7125, Rheodyne) fitted with 20-μl loop. The system consisted of a Constametric III pump (Laboratory Data Control), two in-line filters of 2 and 0.5 μm (Upchurch) were placed before the column TSK G5000 PWXL, 300 mm × 7.6 mm (Toya Soda), the flow-rate of mobile phase, 0.2 *M* ammonium acetate, was 0.3 ml/min. The eluate was passed into a differential refractometer (Refractor Monitor IV, Milton Roy), sensitivity $0.01 \cdot 10^{-3}$ ΔRI and the resulting change in eluate concentration was registered on a recording integrator (SP 4270, Spectra-Physics). For the analysis of vaccine stability a second column, TSK G2500 PWXL, 300 mm × 7.6 mm (Toya Soda) was connected after the TSK G5000 PWXL column.

RESULTS

Pullulans, linear macromolecular polysaccharides that consist of polymers of 1–6-linked α-maltotriose units were used as calibration standards as their molecular weights and molecular weight distribution obtained by ultracentrifugal sedimentation equilibrium measurements are known and they are commercially available. Potassium chloride and a very-high-molecular-weight dextran ($>2 \cdot 10^6$) were used to provide the total permeation volume (V_t) and the exclusion volume (V_0) respectively. The K_D value was calculated using the following equation[7–9]

$$K_D = \frac{V_R - V_0}{V_t - V_0}$$

where V_R = retention volume of the component of interest, V_t = retention volume of a component that has full access to all the pores in the support (total permeation volume) and V_0 = retention volume of a non-retained component (exclusion volume).

Calibration of the two columns Sepharose 4BCL and TSK 5000 PWXL with the pullulans demonstrated a linear relationship between logarithm of molecular mass and K_D, the coefficients of linear correlation were 0.98 and 0.99 respectively. There was no significant difference ($t = 0.77$ with 14 degrees of freedom) between the two sets of calibration data, on both columns the estimated K_D of the pullulan, molecular mass $10.0 \cdot 10^4$ corresponded closely to a K_D of 0.5 (Table I and Fig. 1). To analyse a sample on the Sepharose 4BCL column required at least 3 mg of material and took 320 min compared with 100 μg of material and 35 min on the TSK 5000 PWXL column.

TABLE I

DISTRIBUTION COEFFICIENTS (K_D) OF THE PULLULANS AND MENINGOCOCCAL POLYSACCHARIDES AS DETERMINED ON A SEPHAROSE 4BCL COLUMN AND A TSK 5000 PWXL COLUMN

Experimental conditions as described in the text.

Substance	*Molecular mass* (× 10^4)	K_D	
		Sepharose 4BCL	*TSK 5000 PWXL*
Pullulan 800	85.30	0.09	0.11
Pullulan 400	38.00	0.27	0.23
Pullulan 200	18.60	0.39	0.36
Pullulan 100	10.00	0.49	0.48
Pullulan 50	4.80	0.63	0.60
Pullulan 20	2.37	0.72	0.71
Pullulan 10	1.22	0.78	0.78
Pullulan 5	0.58	0.82	0.84
Meningococcal polysaccharide[a]			
Group A (M)		0.30	0.25
Group C (M)		0.20	0.22
Group Y (M)		0.03	0.14
Group W135 (S)		0.08	0.14
Group A (S)		–	0.25
Group C (S)		–	0.28
Group A (C)		–	0.35
Group C (C)		–	0.29
Group Y (C)		–	0.00
Group W135 (C)		–	0.00

[a] M, S and C refer to different manufacturers.

The current pharmacopoeial specification for the meningococcal polysaccharide vaccine is "Not less than 65% of the group A polysaccharide, 75% of the group C polysaccharide, 80% of the group Y polysaccharide and 80% of the group W135 polysaccharide are eluted before a distribution coefficient (K_D) of 0.5 is reached." Examination of the limited number of samples available on both Sepharose 4BCL column and the TSK 5000 PWXL column showed that agreement was better for the lower-molecular-size polysaccharides, *i.e.* groups A and C than for the higher-molecular-size polysaccharides, *i.e.* groups Y and W135 (Table II). For comparison elution profiles obtained using both chromatographic procedures are shown for group

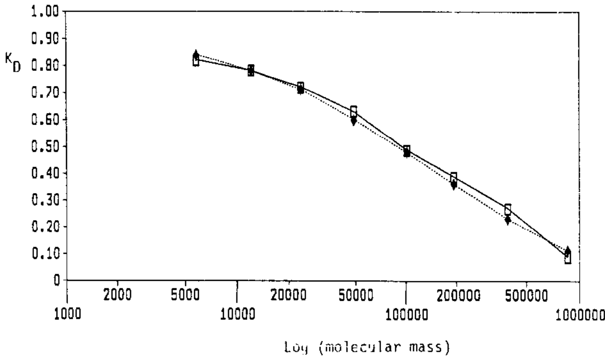

Fig. 1. Calibration profile showing the relationship between K_D and molecular mass of polysaccharides in 0.2 *M* ammonium acetate using (□) Sepharose 4BCL and (◆) TSK 5000 PWXL column.

Fig. 2. Elution profile of meningococcal group C polysaccharide from a Sepharose 4BCL column monitored by refractive index (top), sialic acid content (middle) and from a TSK 5000 PWXL column monitored by refractive index (bottom). Experimental details in text.

Fig. 3. Elution profile of meningococcal group Y polysaccharide from a Sepharose 4BCL column monitored by refractive index (top), sialic acid content (middle) and from a TSK 5000 PWXL column monitored by refractive index (bottom). Experimental details in text.

TABLE II

PROPORTION OF MENINGOCOCCAL POLYSACCHARIDE ELUTING BEFORE THE K_D 0.5 IS REACHED ON A SEPHAROSE 4BCL COLUMN DETERMINED CHEMICALLY AND BY REFRACTIVE INDEX AND (A) TSK 5000 PWXL COLUMN DETERMINED BY REFRACTIVE INDEX

Meningococcal polysaccharide group	*British Pharmacopoeia specification Not less than*	*Sepharose 4BCL*		*TSK 5000 PWXL, RI*
		Chemical	*RI*	
A (M)	65	81	87	86
A (S)		–	–	87
A (C)		–	–	78
C (M)	75	95	94	95
C (S)		–	–	85
C (C)		–	–	85
Y (S)	80	98	96	91
Y (C)		–	–	93
W135 (S)	80	98	95	91
W135 (C)		–	–	90

C and group Y polysaccharides (Figs. 2 and 3). All the samples examined readily complied with the current pharmacopoeial requirement. using the same molecular size criterion samples shown to be satisfactory by GPC were found to be satisfactory when examined by high-performance GPC. Four pneumococcal polysaccharides have been tested by both methods. For three of them the agreement was poor due to their large molecular size and polydisperse nature. In order to stabilise the meningococcal polysaccharides during freeze-drying and subsequent storage an inert carrier, usually lactose, is included in the formulated vaccine. Therefore to monitor the stability of the vaccine a high-performance GPC method is required which separates the low-molecular-mass carrier sugar from the depolymerization products. Connecting the two TSK columns in series gave an excellent separation of both high- and low-molecular-mass carbohydrates (Table III). The plot of K_D against logarithm of the molecular mass

TABLE III

K_D VALUES OF THE PULLULANS AND SUGARS AS DETERMINED ON TSK 5000 PWXL COLUMN CONNECTED IN SERIES WITH A TSK 2500 PWXL COLUMN

Experimental conditions as described in the text.

Substance	*Molecular mass*	K_D	*Substance*	*Molecular mass*	K_D
Threitol	122	0.965	Pullulan 5	5800	0.578
Erythritol	122	0.964	Pullulan 10	12 200	0.507
Glucose	180	0.933	Pullulan 20	23 700	0.442
Mannitol	182	0.920	Pullulan 50	48 000	0.369
Lactose	360	0.862	Pullulan 100	100 000	0.303
Trehalose	378	0.867	Pullulan 200	186 000	0.226
Stachyose	666	0.784	Pullulan 400	380 000	0.143
			Pullulan 800	853 000	0.060

yielded a correlation coefficient of 0.9997 (Fig. 4). Changes in the molecular size distribution of meningococcal group A polysaccharide freeze-dried in the presence of lactose and mannitol then stored at −20 and 37°C for 24 days were readily detectable (Fig. 5).

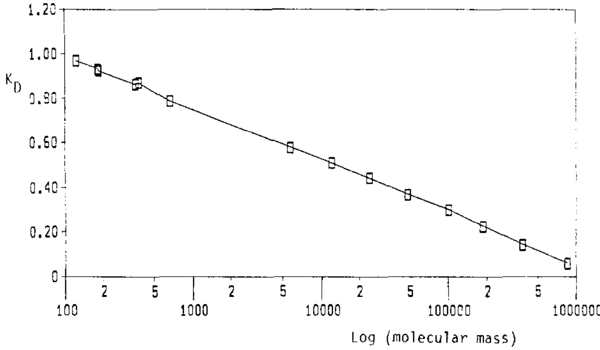

Fig. 4. Calibration profile showing the relationship between K_D and molecular mass of polysaccharides and low-molecular-mass sugars in 0.2 M ammonium acetate using TSK 5000 PWXL and TSK 2500 PWXL columns in series.

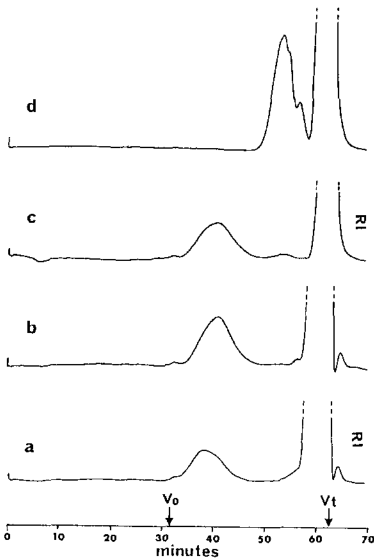

Fig. 5. Elution profile of meningococcal group A polysaccharide from TSK 5000 PWXL and TSK 2500 PWXL columns in series monitored by refractive index. Polysaccharide 0.2 mg and lactose 4 mg kept for 24 days at −20 (a), 37°C (b) and Polysaccharide 0.2 mg and mannitol 4 mg kept for 24 days at −20 (b), 37 (c) and 37°C (d).

CONCLUSIONS

Our results indicate that the current GPC procedure can be replaced by high-performance GPC for the molecular size determination of the individual meningococcal polysaccharides used in vaccines with a significant saving of material and time. The GPC method does allow the chemical characterization of the individual meningococcal group polysaccharide components of a multivalent vaccine. Because of the smaller load necessary for high-performance GPC chemical assay of the individual vaccine components is not feasible. Provided the polysaccharides used in the vaccine are satisfactory, high-performance GPC can be used to check the overall molecular size distribution in the vaccine. Thus it is a extremely convenient method of monitoring changes occurring during the processing and stability trials of meningococcal vaccines. The effect of different carrier sugars upon the stability of the meningococcal group polysaccharides during freeze-drying is being monitored using high-performance GPC. The availability of suitable GPC software packages will enable molecular size distribution changes to be readily quantified. The high-performance GPC column used was not entirely satisfactory for the examination of the large-size pneumococcal polysaccharides but as the working range of these columns is extended it may not be long before the pneumococcal polysaccharides can be examined routinely by high-performance GPC.

REFERENCES

1 A. Galazka, *Bull. WHO*, 60 (1982) 1.
2 *WHO Techn. Rep. Ser., No. 594*, World Health Organization, Geneva, 1976, pp. 19 and 50.
3 *WHO Techn. Rep. Ser., No. 610*, World Health Organization, Geneva, 1977, p. 52.
4 *British Pharmacopoeia*, HMSO, London, 1988, p. 1058.
5 P. S. Chen, T. Y. Toribara and H. Warner, *Anal. Chem.*, 28 (1956) 1756.
6 L. Svennerholm, *Biochim. Biophys. Acta*, 24 (1957) 604.
7 G. K. Ackers, *Biochemistry*, 3 (1964) 7213.
8 R. M. Alsop and G. J. Vlachogiannis, *J. Chromatogr.*, 246 (1982) 227.
9 D. M. Hittner and M. K. Cowman, *J. Chromatogr.*, 402 (1987) 149.