Journal of Chromatography, 565 (1991) 416–423
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5777

Short Communication

Novel affinity chromatographic system for the single-step purification of glycosaminoglycans from complex systems using volatile buffers

B. A. HODSON

Centre for Reproductive Biology, 37 Chalmers Street, Edinburgh EH3 9EW (U.K.)

D. S. PEPPER

SNBTS H.Q. Laboratories, 2 Forrest Road, Edinburgh EH1 2QN (U.K.)

and

J. DAWES*

Heart Research Institute, 145 Missenden Road, Camperdown, NSW 2050 (Australia)

(First received August 28th, 1990; revised manuscript received December 4th, 1990)

ABSTRACT

A new system for the isolation and purification of glycosaminoglycans (GAGs) and related molecules from complex systems such as plasma is described. Affinity chromatography which exploits the very high affinity between the polymeric base Polybrene and sulphated polysaccharides was used. A novel volatile buffer system composed of ammonium formate and formic acid, which allows the complete recovery of samples, was developed, and elution conditions were optimised for the separation and purification of GAGs of different charge densities. Using this system the losses associated with dialysis and desalting, frequently necessary preliminaries to further analysis, are avoided.

INTRODUCTION

Glycosaminoglycans (GAGs) and related compounds are highly negatively charged linear polysaccharides which in solution tend to adopt extended configurations on account of the mutual repulsion of their many carboxyl and sulphate ester groups. The linear polymeric base Polybrene (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide), of average molecular mass 10 000, has a very high affinity for GAGs [1,2] with multiple interactions per GAG molecule. This provides the basis for an affinity material with superior discrimination to those using monovalent bases such as simple ion exchangers.

To elute GAGs from Polybrene columns high-ionic-strength solutions are

0378-4347/91/$03.50

needed and to provide this buffered sodium chloride solution may be used. However, this eluent gives a product with a high salt content which interferes with further analytical procedures including the sensitive GAG assay developed by Dawes *et al.* [1], degradation by specific glycosidases, and identification of constituent saccharides by NMR. Dialysis and other routine desalting manipulations markedly reduce the yield of GAG. An alternative system is described here using ammonium formate and formic acid, the components of which can be removed under vacuum to provide complete recovery of sample; the method is particularly valuable for the purification and analysis of naturally occurring GAGs which may be isolated in relatively small quantities.

EXPERIMENTAL

Reagents

Sephacryl S300 was purchased from Pharmacia U.K. and Polybrene, heparin and 1,4-butanedioldiglycidyl ether (1,4-BDDGE) from Sigma U.K. Sulpho-N-succinimydyl-3-(4-hydroxyphenyl)propionate (sulpho-SHPP) was from Pierce and Warriner (Chester, U.K.). Dermatan sulphate MF701 Batch 48 was a generous gift of Dr. F. Gianese (Mediolanum Farmaceutici, Milan, Italy), and pentosan polysulphate SP54 was a gift of the late Dr. T. Halse (Benechemie, Munich, Germany).

Epoxy activation of solid-phase support

The solid-phase material found to be most suitable as the basis for Polybrene anion-exchange columns was the dextran–bisacrylamide copolymer Sephacryl, which was epoxy-activated by the method of Sundberg and Poráth [3]. Equal volumes of swollen gel beads (33%, v/v), 1,4-BDDGE and 0.6 *M* NaOH were gently mixed by end-over-end rotation at 20°C for 20 h. The activated gel beads were then washed very thoroughly with warm (40°C) distilled water in a sintered-glass funnel until all traces of unreacted 1,4-BDDGE had been removed.

Covalent coupling of Polybrene to epoxy-activated Sephacryl beads

The reaction was carried out as follows by a modification of the method of Dawes *et al.* [1]. Equal volumes of swollen activated gel beads and 2% (w/v) Polybrene in 1 *M* Na_2CO_3 (pH 11.4) were mixed and the pH was adjusted to 12.5 ± 0.25 by the addition of a NaOH (40%, w/v) solution. The reactants were gently mixed by end-over-end rotation for 48 h at 20°C, washed in a sintered-glass funnel with a large volume of distilled water until near neutral pH, and finally suspended in 20 m*M* sodium citrate buffer pH 6.7 containing 0.02% NaN_3. The Polybrene–Sephacryl was stable between pH 2.5 and 13 and showed minimal leaching of free Polybrene. The final amount of Polybrene coupled was in the region of 10 mg Polybrene per ml of swollen gel, as determined by Naphthol blue black titration. A 0.005% (w/v) solution of Naphthol blue black (Sigma) in water

was mixed with 200 μl of a 1:1 (v/v) gel slurry. Dye solution was added and mixed repeatedly until approximately 50% remained in solution as measured by adsorption at 500 nm. The amount of dye bound was expressed as μmol/ml of gel. The functional binding capacity of the gel for GAGs depends on the accessible pore volume of the gel for the molecules under investigation, but for Sephacryl S400 it was in the region of 5 mg heparin per ml.

Preparation of [^{125}I]heparin

^{125}I-Labelled heparin (50 ng) was used throughout this study as an internal standard which could be monitored conveniently by γ-counting. It was prepared by derivatizing heparin with sulpho-SHPP essentially according to the method of Dawes and Pepper [4], and iodinating the derivative using the chloramine T method of Greenwood *et al.* [5].

Assay of GAGs

GAG concentrations were measured using the Azure A metachromatic assay of Lam *et al.* [6].

RESULTS

Elution of heparin with volatile buffers

The efficacy of prospective volatile eluents was determined in relation to that of the system characterised by Dawes [2]. [^{125}I]Heparin (50 ng) was loaded onto 4 ml of Polybrene–Sephacryl S400, and after an initial wash with 5 column volumes of 20 m*M* sodium citrate pH 6.7 it was eluted with a linear 0–2 *M* NaCl gradient in 20 m*M* sodium citrate pH 6.7 for 30 column volumes. The column was then

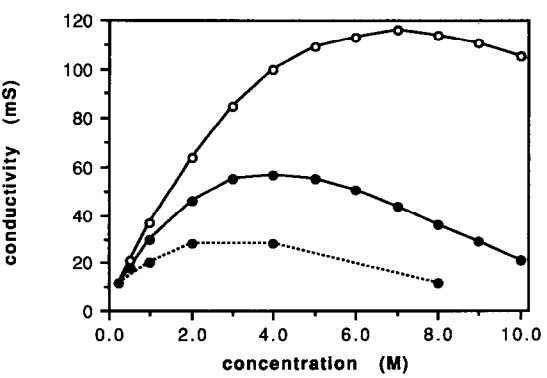

Fig. 1. Effect of concentration on the conductivity of solutions of ammonium acetate in water (—●—) and in 20% dimethylsulphoxide in water (···●···), and of ammonium formate in water (—○—). All measurements were carried out at ambient temperature.

washed with 3 *M* NaCl to elute residual [^{125}I]heparin. The peak of [^{125}I]heparin eluted at a conductivity of 40 mS following application of a 0–2 *M* NaCl gradient, and none remained on the column. Equally effective elution was obtained at 55 mS when the gradient contained NH_4Cl rather than NaCl. However, NH_4Cl was insufficiently volatile to constitute an ideal eluent, and more volatile anions were therefore substituted for Cl^-. The conductivities of increasing concentrations of ammonium bicarbonate, ammonium acetate and ammonium formate in water were measured. Solutions of ammonium bicarbonate are saturated at 2.2 *M*, which gave a maximum conductivity of 55 mS; with the other salts the conductiv-

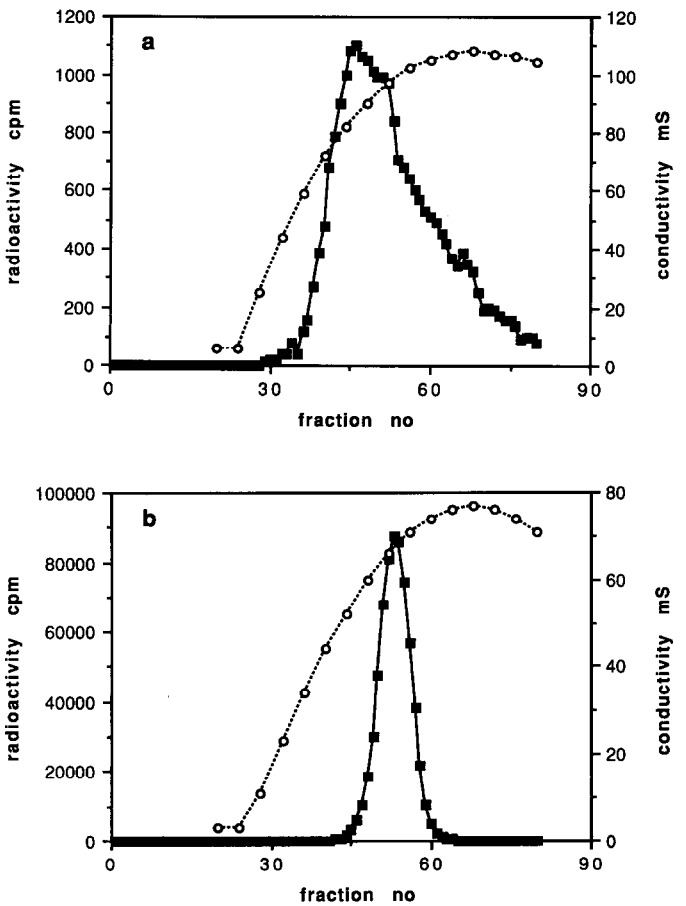

Fig. 2. Elution of [^{125}I]heparin from Polybrene–Sephacryl with volatile buffers. [^{125}I]Heparin (50 ng) in 0.05 *M* ammonium formate pH 6.8 was applied to 4 ml of Polybrene–Sephacryl S400. An initial wash of 40 ml of 0.05 *M* ammonium formate pH 6.8 (twenty 2-ml fractions) was followed by elution with (a) a 120-ml 0–10 *M* ammonium formate gradient or (b) a double gradient of 0–6 *M* ammonium formate and 0–6 *M* formic acid; 2-ml fractions were collected. The pH fell rapidly to 3.5 over the first 10 ml of the double gradient, and remained constant thereafter. Radiolabelled heparin (■) was monitored by γ-counting; the conductivity (○) of every fourth fraction was also measured.

ity reached a peak and then declined with further increases in concentration (Fig. 1). On the basis of earlier evidence that dimethylsulphoxide (DMSO) decreased the affinity of GAGs for Polybrene [1], the effect of DMSO on heparin elution was tested. Incorporation of 20% DMSO into the elution buffers decreased the maximum conductivity attainable with each ammonium salt by 50% (illustrated for ammonium acetate in Fig. 1). Gradients were constructed to the maximum conductivity of each salt. A 0–2.2 *M* NH_4HCO_3 gradient failed to elute heparin, as did 0–3 *M* ammonium acetate, but a broad peak containing all the [^{125}I]heparin was eluted by a 0–6 *M* ammonium formate gradient within the conductivity range 80–100 mS (Fig. 2a); heparin was not eluted by any of the ammonium salt gradients containing 20% DMSO. The width of the peak obtained from the ammonium formate gradient indicated that these elution conditions would discriminate poorly between different GAGs, but it was found that incorporating a 0–50% formic acid gradient in the elution buffer eliminated this problem, giving a sharp peak of [^{125}I]heparin at 65 mS (Fig. 2b).

Elution conditions for GAG samples in plasma or serum

The elution conditions were optimised for GAGs in complex biological fluids, exemplified by plasma and serum. An initial wash of 5 column volumes of 0.05 *M* ammonium formate (pH 6.8) was followed by 2.5 column volumes of 0.1 *M* ammonium formate adjusted to pH 4 with formic acid. All plasma proteins, monitored by absorbance at 280 nm, eluted either in the void volume or during these preliminary washing steps. A linear gradient in 30 column volumes of ammonium formate and formic acid was then used to elute the GAG. The composition of the gradient was selected according to the degree of sulphation of the GAG under investigation. For heparin (31% sulphate), heparan sulphate and dermatan sulphate (16–20% sulphate) the starting buffer was 0.1 *M* ammonium formate adjusted to pH 4.0 with 98–100% formic acid, and the final buffer was 6 *M* ammonium formate in 6 *M* formic acid. A typical isolation of dermatan sulphate from plasma is illustrated in Fig. 3a; this material, which contained 20% sulphate, eluted at 45 mS. For more highly sulphated compounds higher concentrations of formic acid were required in the final buffer, with pentosan polysulphate (48% sulphate) being eluted at about 67 mS with 6 *M* ammonium formate in 12 *M* formic acid (Fig. 3b).

The ammonium formate–formic acid could be completely removed from the column fractions in a centrifugal evaporator at a rotor temperature of 45°C and a pressure of less than 5 mbar. Pooled fractions may also be recovered by the use of a freeze-drier with the flask containing the fractions heated to 40°C during the secondary drying stage. The pH of the ammonium formate–formic acid slurry tended towards pH 6 as evaporation/sublimation proceeded. However, the high buffering capacity of the ammonium formate protected the GAG during this process as shown by re-chromatography of [^{125}I]heparin tracer, which was recovered by elution from immobilised Polybrene at the same molarity of NaCl as native heparin.

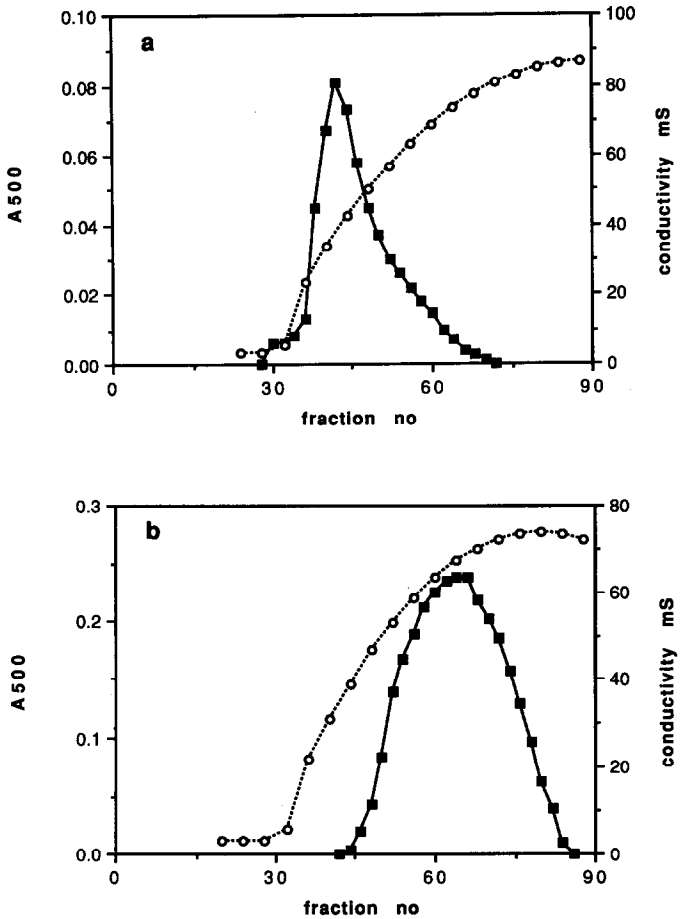

Fig. 3. Purification of (a) dermatan sulphate MF701 and (b) pentosan polysulphate SP54 by affinity chromatography on Polybrene–Sephacryl S400 and elution with volatile buffers. Dermatan sulphate or pentosan polysulphate (3 mg) were added to 1 ml of human plasma and applied to 4 ml of Polybrene–Sephacryl S400. An initial wash of 40 ml of 0.05 *M* ammonium formate pH 6.8 was followed by 20 ml of 0.1 *M* ammonium formate pH 4.0 and then by a linear double gradient of 120 ml of 0–6 *M* ammonium formate containing (a) 0–6 *M* and (b) 0–12 *M* formic acid at a constant pH of 3.5; 2-ml fractions were collected. Sulphated polysaccharides were measured by absorbance at 500 nm (■) after Azure A assay [6], and conductivity (○) was also recorded.

DISCUSSION

Technology for improved purification and subsequent analysis of GAGs from biological samples is increasingly required as these highly negatively charged polysaccharides are implicated in an ever broader range of biological functions. Systems are needed no only to purify naturally occurring GAGs from healthy and diseased tissues, but also to investigate fractionation of exogenous ther-

apeutic GAGs *in vivo* which can result in modification of their predicted efficacy with time. GAGs are heterogeneous in composition with a spectrum of charge, size and biological activity present in all isolates. Since only a portion of molecules may possess biological acitivity as exemplified by the 25–35% (w/w) of commercial heparin having anticoagulant activity [7], any uncontrolled fractionation of the material during purification could alter the properties of the isolate. Such fractionation can occur during dialysis, with small and highly charged molecules being preferentially lost. In our hands the losses on dialysis of GAGs with an average molecular mass of 6000 can be as high as 70% when using a Spectropore S1 membrane with a molecular mass cut-off at 3500 dalton (unpublished results). These high losses result from the ability of the "rod-like" molecules to stream through the membrane. To avoid such losses a system using volatile buffers was devised.

We have shown previously [1,2] that immobilised Polybrene is a highly effective affinity matrix for GAGs and related molecules such as dextran sulphate, and that purification from complex mixtures can be achieved by elution with a NaCl gradient. We have now developed an elution system which is similarly effective, but consists of a double gradient of ammonium formate and formic acid. Although initial development of the elution system was based on the maximum conductivities of solutions of different ammonium salts, it became clear that this was only one of the parameters affecting disruption of Polybrene–GAG complexes, even at neutral pH. Thus the conductivity required to elute heparin was much less for ammonium chloride than for ammonium formate. The superposition of a gradient of formic acid together with a lower pH resulted in an elution system which could be applied to the purification of all naturally occurring GAGs as well as related synthetic materials, and would fractionate GAGs of different sulphate contents. The eluent was composed of fully volatile constituents which could be subsequently removed by centrifugal evaporation or freeze-drying, permitting further analysis of the GAGs by methods which are sensitive to high salt content such as the quantitative competitive binding assay [1], specific enzymic degradation and NMR analysis of constituent saccharides.

ACKNOWLEDGEMENTS

This research was supported in part by Medical Research Council project Grant No. G86 07394 SA. We are very grateful to Dr. Francesco Gianese of Mediolanum Farmaceutici and the late Dr. T. Halse of Benechemie for gifts of dermatan sulphate and pentosan polysulphate, respectively.

REFERENCES

1 J. Dawes, C. V. Prowse and D. S. Pepper, *Thromb. Haemostasis*, 54 (1985) 630.
2 J. Dawes, *Anal. Biochem.*, 174 (1988) 177.

3 L. Sundberg and J. Poráth, *J. Chromatogr.*, 90 (1974) 87.
4 J. Dawes and D. S. Pepper, *Thromb. Res.*, 14 (1979) 845.
5 F. C. Greenwood, W. M. Hunter and J. S. Glover, *Biochem. J.*, 89 (1963) 114.
6 L. H. Lam, J. E. Silbert and R. D. Rosenberg, *Biochem. Biophys. Res. Commun.* 69 (1976) 570.
7 M. Hook, I. Bjork, J. Hopwood and U. Lindahl, *FEBS Lett.*, 66 (1976) 90.