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Note

Continuous-flow monitoring of hexuronic acid by carbazole reaction during gel filtration of proteoglycans in urea solutions

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Gel filtration is one of the methods frequently employed for purification and fractionation of proteoglycans[1–3]. Since proteoglycans are known to form large aggregate structures under associative conditions[4,5], gel filtration can be used to study aggregation. *In vitro*, this association is reversible and is largely dictated by the solvent. Proteoglycans are usually isolated from many tissues by extraction with dissociative solvents such as 4 *M* guanidine hydrochloride or 6 *M* urea[5,6], followed by cesium chloride isopycnic centrifugation and/or gel filtration. Isolation of aggregates by gel filtration is achieved by using salt solutions of low concentration, *e.g.*, 0.4 *M* guanidine · HCl, while 4 *M* guanidine · HCl or 6 *M* urea is used for isolation of proteoglycan monomers. After gel filtration, fractions generally are dialyzed to remove salts and then monitored for hexuronic acid by carbazole reaction. We recently described a procedure for continuous-flow analysis of proteoglycans during gel filtration in guanidine · HCl by an orcinol–sulfuric acid reaction[7]. This is a general reaction for sugars and is not specific for uronic acids.

This report describes a procedure for continuous-flow monitoring of proteoglycans by a carbazole–sulfuric acid reactions using the Technicon sugar chromatography system. The proteoglycans were eluted from gel filtration by a dissociative solvent, 6 *M* urea.

EXPERIMENTAL

Materials

Separhose CL-6B was purchased from Pharmacia (Piscataway, NJ, U.S.A.). Reagent-grade (ACS grade) sulfuric acid was obtained from DuPont (Wilmington, DE, U.S.A.), potassium tetraborate and urea from J. T. Baker (Phillipsburg, NJ, U.S.A.), and carbazole from Eastman Organic Co. (Rochester, NY, U.S.A.). Glu-

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curonolactone was purchased from Corn Products Refineries (New York, NY, U.S.A.). Bovine aorta proteoglycan was available in the laboratory from other studies[8–10]. Glycosaminoglycan standards were donated by Drs. J. A. Cifonelli and M. B. Matthews, University of Chicago.

Sulfuric acid–borate reagent. Potassium tetraborate (19.1 g) was dissolved in 200 ml distilled water, and to this solution 2300 ml of concentrated sulfuric acid (97%) were carefully added in the cold.

Carbazole reagent. Carbazole (1.0 g) was dissolved in 1 l of 95% ethanol and stored at 4°C.

Glucuronolactone standard solution. Standard solutions of glucuronolactone were prepared either in distilled water or in different concentrations of urea in 0.5 *M* sodium acetate, pH 5.8.

Methods

Isolation of proteoglycans from bovine aorta. Proteoglycans were isolated from bovine aorta intima as previously described[9–11]. Proteoglycans were extracted with 4 *M* guanidine · HCl in the presence of protease inhibitors. The extract was clarified by centrifugation, and the density was adjusted to 1.33 g/ml by the addition of cesium chloride. The solution was centrifuged at 100 000 *g* for 19 h at 4°C. The bottom two-fifths of the gradient was adjusted to a density of 1.46 g/ml with cesium chloride recentrifuged as before. Fractions of the bottom two-fifths gradient were pooled, dialyzed and lyophilized.

Automated gel filtration procedure. The flow diagram of the gel filtration procedure is illustrated in Fig. 1. A chromatographic column (76 × 2 cm I.D., 3400 Series; Glenco Scientific, Houston, TX, U.S.A.) filled with Sepharose CL-6B was used. The flow-rate was kept constant at 0.5 ml/min by negative pressure, *i.e.*, by passing the column outlet tube through the proportioning pump manifold. Pump tubing sizes are shown in the figure. Colorimeter and recorder settings were the same as those described previously[12,13]. A 530-nm filter was used in the colorimeter.

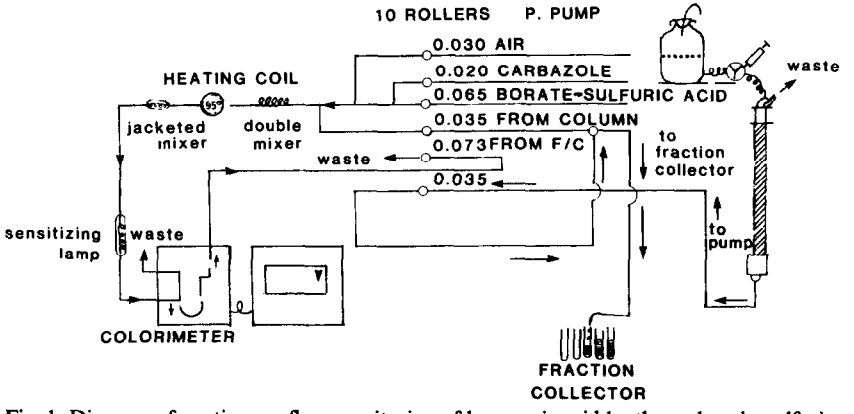

Fig. 1. Diagram of continuous-flow monitoring of hexuronic acid by the carbazole–sulfuric acid reaction in the Technicon sugar chromatography system. All connections are with 1.6 mm glass–0.090 in. Acidflex sleeve; tube size is given in inches. Colorimeter is equipped with a 15-mm tubular flow cell (F/C) and a 530-nm filter.

The analyzer was first primed with 95% ethanol before adding carbazole reagent. If carbazole reagent is used directly for priming, a possibility exists of precipitation of carbazole in the tubing and this would block the pumping of the reagent. Precaution also should be taken that the carbazole reagent should mix with sulfuric acid before it joins the inflowing sample solution in the manifold (*i.e.*, carbazole reagent should be introduced in the system when the other reagents are being pumped freely into the system).

Prior to gel filtration of proteoglycans, the Sepharose column was equilibrated with 6 *M* urea in 0.5 *M* sodium acetate, pH 5.8. Samples of proteoglycans or glycosamidoglycan were dissolved in this buffer and added to the top of the column. The column was then eluted with buffer and the effluent was monitored continuously in the sugar analyzer by carbazole–sulfuric acid.

Determination of glucuronic acid. Manual determination of glucuronic acid in distilled water or in urea solutions was performed by the procedure of Bitter and Muir[14]. Solutions of different concentrations of glucuronic acid in 0.5 *M* sodium acetate, pH 5.8, and in 6 *M* urea in 0.5 *M* sodium acetate, pH 5.8, were analyzed by the autoanalyzer. Glucuronic acid samples were introduced through the sample inlet of the manifold and analyzed by the carbazole reaction. The areas of the peaks on the recorder chart were calculated by triangulation.

RESULTS AND DISCUSSION

Earlier, Ford and Baker[15] reported an automated procedure for hexuronic acid determination by carbazole–sulfuric acid reaction using the Technicon sugar analyzer. In this method carbazole was dissolved in sulfuric acid–borate and pumped into the system. Unfortunately, this reagent is not stable even at 4°C and gradually turns yellow in 4–6 h. Since many gel filtration procedures require long durations, we found that the procedure is not suitable for continuous-flow monitoring during chromatography. In the present method we introduced carbazole reagent and sulfuric acid–borate reagent separately into the system and allows them to mix with each other before mixing with uronate samples. Both reagents are stable indefinitely when stored at 4°C. The manifold in the autoanalyzer was modified to accommodate the changes (Fig. 1).

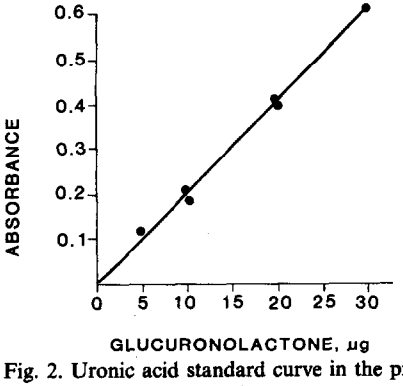

Fig. 2. Uronic acid standard curve in the presence or absence of 6 *M* urea. Both curves were identical. Absorbance was measured at 530 nm.

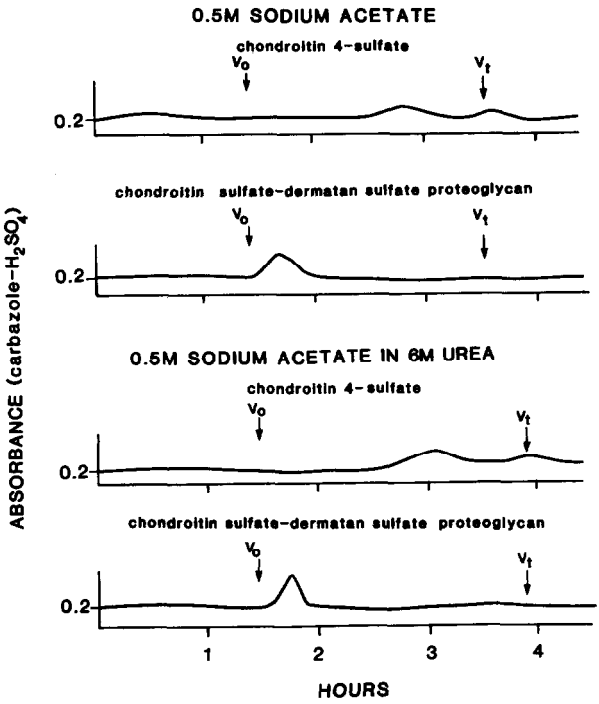

Fig. 3. A comparison of gel filtration profiles of chondroitin 4-sulfate and a chondroitin sulfate–dermatan sulfate proteoglycan from bovine aorta in the presence or absence of 6 *M* urea in sodium acetate monitored by the carbazole–sulfuric acid reaction. Absorbance was measured at 530 nm.

Fig. 2 illustrates the effect of urea on the color absorbance of carbazole reaction of glucuronic acid[14] in a test tube. Urea in 0.5 *M* sodium acetate, even at a concentration of 6 *M*, did not suppress the color. However, when protease inhibitors were included in the solvent along with 6 *M* urea, an approximately 7% decrease in absorbance occurred at a concentration of 60 μg/ml uronic acid.

Glucuronic acid solutions (either in sodium acetate or in 6 *M* urea in sodium acetate) gave peak areas of identical values when analyzed in the autoanalyzer by the procedure described. This suggests that urea does not interfere in the method. Analyses of ten identical samples of glucuronic acid in 6 *M* urea in sodium acetate gave a coefficient of variation of 3%. When known concentrations of glucuronic solution in 6 *M* urea and 0.5 *M* sodium acetate were analyzed in the autoanalyzer, the areas of the peaks on the recorder trace were proportional to the glucuronic acid concentration, suggesting the method is suitable for monitoring proteoglycans or glycosaminoglycans.

Fig. 3 illustrates the gel filtration profiles of chondroitin 4-sulfate and a chondroitin sulfate–dermatan sulfate proteoglycan from bovine aorta in sodium acetate and in 6 *M* urea in sodium acetate. The peaks in chromatograms developed with 6 *M* urea in sodium acetate are somewhat sharper than those developed with sodium acetate alone, but the areas of the peaks in both buffers were the same. Chondroitin sulfate and the chondroitin sulfate–dermatan sulfate proteoglycan peaks eluted from

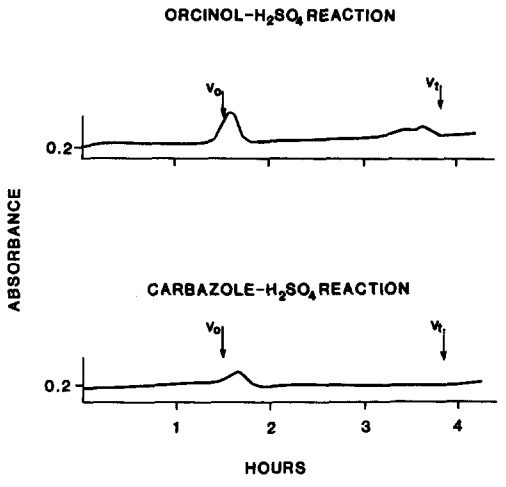

Fig. 4. Comparison of orcinol–sulfuric acid reaction (measured at 420 nm) and carbazole–sulfuric acid reaction (measured at 530 nm) for monitoring hexuronic acid during gel filtration of a chondroitin sulfate–dermatan sulfate proteoglycan from bovine aorta intima.

the column somewhat slower in urea. Our observations suggest that the procedure is adequately sensitive for monitoring hexuronic acid during chromatography of proteoglycans in 6 *M* urea.

Fig. 4 shows a comparison of gel filtration profiles of a partially purified sample of chondroitin sulfate–dermatan sulfate proteoglycan continuously monitored during gel filtration by carbazole–sulfuric acid reaction and by orcinol–sulfuric acid reaction. Both chromatograms show one major peak near the void volume. The peak in the chromatogram analyzed by carbazole–sulfuric acid had a smaller area than the same peak in the chromatogram analyzed by orcinol–sulfuric acid. In the orcinol–sulfuric acid analyzed chromatogram a second peak of a much lower molecular weight appeared, which was absent in the carbazole–sulfuric acid monitored run. This documents the increased specificity of the carbazole–sulfuric acid reaction over the orcinol–sulfuric acid reaction.

Carbazole–sulfuric acid is at least five times as sensitive to hexuronic acid than orcinol–sulfuric acid. The differences in the areas of peaks between the chromatograms are likely due to either short oligosaccharide chains present on the core protein of the proteoglycan or contaminant glycoproteins in the preparation. Although the carbazole–sulfuric acid reaction is specific for hexuronic acid, neutral sugars, particularly at high concentrations, will produce some color.

In conclusion, the method described affords a procedure for monitoring proteoglycans during gel fitlration by a color reaction specific to hexuronic acids and will significantly aid isolation of proteoglycans.

ACKNOWLEDGEMENTS

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