THIN-LAYER CHROMATOGRAPHY AND THE RAPID IDENTIFICATION OF COMMON ACIDIC GLYCOSAMINOGLYCANS

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The chemical structures of the common acidic glycosaminoglycans (mucopolysaccharides*) have only recently been established with any certainty and there are few accepted methods for the rapid examination and identification of compounds belonging to this class. A major difficulty in chromatographic and electrophoretic studies[1] on these substances results from the wide variations in molecular size of otherwise pure polysaccharide samples. The chain-length of any one the polymers may depend both on the tissue of origin and the means of detachment from the protein to which it is bound *in vivo*.

Qualitative analysis of mixtures of the polymers is possible by chromatography on silicated glass paper[2] or filter paper[3], both techniques requiring multiple elution steps. The present paper describes the adaptation of these methods to thin layers of silica gel and their extension, by means of specific degradation techniques and selective spray reagents, to provide a simple means for preliminary examination of uncharacterised MPS material.

MATERIALS AND METHODS

Acidic glycosaminoglycan reference compounds

Hyaluronate preparations from human umbilical cord were commercial products (Seravac Ltd., and Sigma Chemical Co.). Keratan sulphate from bovine cornea was a gift from Dr. J. D. GREGORY, Rockefeller Institute, New York and a preparation of oversulphated keratan sulphate was obtained from shark cartilage[4]. Heparin (120 and 150 I.U. of anticoagulant activity per mg) were commercial preparations (Evans Medical Co.). Heparan sulphate from human aorta and the urine of patients with Hurler's Syndrome were kindly given by Dr. HELEN MUIR of the Medical Research Council, Mill Hill, London, who also gave a purified sample of the crude heparan sulphate supplied by the Upjohn Company, Michigan. Chondroitin 4-sulphate preparations were derived from fin-whale cartilage[4] and pig nasal septum (Evans Medical Co.). Chondroitin 6-sulphate from human intervertebral disc was a gift from Dr. E. BUDDECKE, Physiologisch-chemisches Institut, University of Tübingen. A further sample of chondroitin 6-sulphate together with pigskin dermatan sulphate

* Abbreviations: MPS = acidic glycosaminoglycans; CPC = cetylpyridinium chloride; HA = hyaluronate; C-4-S = chondroitin 4-sulphate; C-6-S = chondroitin 6-sulphate; DS = dermatan sulphate; HEP = heparin; HS = heparan sulphate; KS = keratan sulphate.

was donated by Dr. E. A. DAVIDSON, Duke University, North Carolina. β-Heparin (dermatan sulphate) was a gift from the late Dr. A. WINTERSTEIN of Hoffman-LaRoche, Basle.

Preparation of thin-layer plates

A slurry of plain silica gel was prepared from 30 g of Whatman SG-41 and 65 ml water and this was applied to five 20 × 20 cm glass plates using a Unoplan Leveller (Shandon and Co.) with the spreader set at 250–300 μ. The plates were dried at 110° for 45 min and stored over anhydrous silica gel.

Plates of Whatman SG-41 impregnated with cetylpyridinium chloride required 80 ml of water containing 0.2 g of CPC (L. Light and Co.). These plates were dried at room temperature.

Chromatographic techniques

Samples of 1–2 μl of 0.5 % aqueous solutions of the reference polysaccharides, in the form of their sodium or potassium salts, were applied with a graduated micropipette. Chromatographic development of the CPC-impregnated plates was achieved simply by standing the glass plate with its lower edge in a trough containing the salt solution. Development of plates using volatile solvents was performed using a saturation chamber as described by DAVIES[5]. The edge of the coated plate was immersed in the developing solvent so that the liquid level was approximately 8 mm below that of the applied spots. Development was allowed to proceed for 30–60 min. On finishing a run the plates were dried in a horizontal position for 30–60 min and sprayed after cooling in air for 5–10 min.

Procedures for the degradation of specific MPS

(*a*) *Hyaluronidase digestion.* To 1 volume of a 0.5 % aqueous solution of reference polysaccharide in the form of its sodium or potassium salt was added 0.2 volume of potassium acetate–acetic acid buffer, pH 5.0, containing potassium chloride and approximately 10,000 international units of testicular hyaluronidase activity per ml, such that the resultant incubation mixture was 0.1 *N* in potassium acetate and 0.15 *N* in potassium chloride. When naturally occurring mixtures of polysaccharides were being examined these were at an overall concentration of 1 % before addition of the buffered enzyme. The mixture was stoppered and incubated at 37° overnight. In order to distinguish the products from chondroitin 4- and 6-sulphates, the hyaluronidase preparation must be free from any enzymic activity which would cause further degradation of oligosaccharides liberated by the polysaccharase. A relatively pure commercial preparation from ovine testes (Wyeth Co.) of 1,200 international units per mg was found to be adequate for this purpose.

After 16–18 h incubation the enzymic digestion was stopped (and solutes converted to free-acid forms) by addition of excess (approximately 200 mg per ml) of air-dried Dowex 50W × 8 (H^+ form, 200–400 mesh). After thorough shaking and centrifugation to remove suspended matter the solution was spotted directly for chromatographic detection of any oligosaccharides liberated during the incubation.

(*b*) *Nitrous acid degradation*[6]. To 1 volume of the acidified hyaluronidase digest obtained in (a) above (or untreated polysaccharide solution after addition of hydrochloric acid to a final concentration of 0.25 *N*) was added 0.1 volume of 4 % aqueous

sodium nitrite. The mixture was incubated at 37° for 1 h before examination of the products for undegraded polymer.

Spray reagents

The application of these sprays to MPS before and after degradative treatment is summarised in Table I.

(*a*) *Metachromatic spray.* The dried plate was sprayed lightly with a saturated solution of Toluidine Blue or Azure A in a mixture of acetone, ethanol and water (1:1:1, v/v). The background was cleared by attaching a wick of Whatman No. 17 paper (by means of a rubber band) and performing ascending development with acetone.

(*b*) *Orcinol–sulphuric acid.* The plate was sprayed with a 1 % solution of orcinol in aqueous sulphuric acid (1:1, v/v) and then heated to 120° until the characteristic colours started to appear. Further heating caused each spot to darken so the initial shade was noted before heating was continued.

This corrosive spray permits detection of small quantities of polysaccharide on silica gel layers in cases where the metachromatic stain is insufficiently sensitive. This is particularly valuable in the case of keratan sulphate (*cf.* CASTOR AND DORSTEWICZ[3]) which was readily distinguished from other MPS because: (1) of the relative ease with which its characteristic pink colour appeared on heating, and (2) after cooling a sprayed plate in the atmosphere, all spots except that due to keratan sulphate faded to pale yellow or grey leaving it as a dark spot.

(*c*) *Nitrous acid–indole.* The plate was sprayed when cool with a freshly mixed solution of nitrous acid (0.5 *N* sodium nitrite and 2 *N* hydrochloric acid, 1:1, v/v). After standing at room temperature for 15–20 min the plate was sprayed with a mixture of 1 % ethanolic indole and phosphoric acid (10:1, v/v). The 2,5-anhydro-sugar residues, produced only from hexosamine residues with free or N-sulphated amino groups, yield a yellow-orange colour with the indole reagent after heating at 120° for about 10 min.

This spray is an adaptation of the method of LAGUNOFF AND WARREN[7] for estimation of N-sulphated aminopolysaccharides.

(*d*) *Naphthoresorcinol spray.* The naphthoresorcinol–phosphoric acid mixture as described by RANDERATH[8] was used to detect oligosaccharides liberated by hyaluronidase digestion. Initial colours are characteristic of the reducing-sugar unit but it was necessary to heat the plate for as long as 20 min at 120° to reveal, as grey spots, oligosaccharides present in low concentration. It was found possible to infer the presence of hyaluronidase-susceptible polysaccharide when present at levels lower than 0.1 % in the initial digestion mixture.

(*e*) *Morgan-Elson spray.* This was used to detect oligosaccharides, produced by hyaluronidase digestion, in which the terminal N-acetylhexosamine residue is unsubstituted in the 4-position (*cf.* MATHEWS AND INOUYE[9]). The version described by SALTON[10] for paper chromatograms was used except that the dried plate was first sprayed with 0.05 *N* sodium borate, pH 10, and heated at 120° for 15 min. It was then sprayed with the acidic *p*-dimethylaminobenzaldehyde solution and heated at 120°. The purple colour, though strong, faded quickly so a control spot of 2 μg of N-acetyl D-glucosamine was added routinely to these plates.

TABLE I

COLOUR REACTIONS OF ACIDIC GLYCOSAMINOGLYCANS ON LAYERS OF SILICA GEL

Treatment	*HA*	*KS*	*C-4-S*	*C-6-S*	*DS*	*HS*	*HEP*
Orcinol-sulphuric acid spray	Purple-grey	Pink	Purple-grey	Purple-grey	Purple-grey	Yellow-brown	Yellow-brown
Nitrous acid–indole spray	—	—	—	—	—	Orange	Orange
Hyaluronidase digestion							
Then:							
(i) Morgan-Elson spray	Purple	—	—	Purple	—	—	—
(ii) Naphthoresorcinol spray	Brown	—	Pink	Brown	—	—	—
(on further heating)	Grey	—	Grey	Grey	—	—	—
Hyaluronidase digestion and nitrous acid treatment							
Then:							
(i) Metachromatic spray	—	Weakly positive	—	—	Strongly positive	—	—
(ii) Indole spray	—	—	—	—	—	Orange	Orange

RESULTS

Detection of hyaluronate, chondroitin 4-sulphate and chondroitin 6-sulphate

Provided the testicular hyaluronidase preparation does not contain substantial quantities of β-glucuronidase, the major product of prolonged digestion of hyaluronate or chondroitin 4- or 6-sulphate is the corresponding tetrasaccharide[11]. The other common MPS are not attacked by the testicular enzyme and, under the experimental conditions described here, do not appreciably inhibit its action. When 1 μl portions from overnight digests containing at least 1 mg of digestible polysaccharide per ml are chromatographed only one mobile chromatographic spot with reducing-sugar properties is produced by each of the three polymers. The parent polysaccharides are identified by a combination of R_F value and colour reaction with the Morgan-Elson and naphthoresorcinol sprays (Fig. 1). The sprays can be applied in sequence to the same plate.

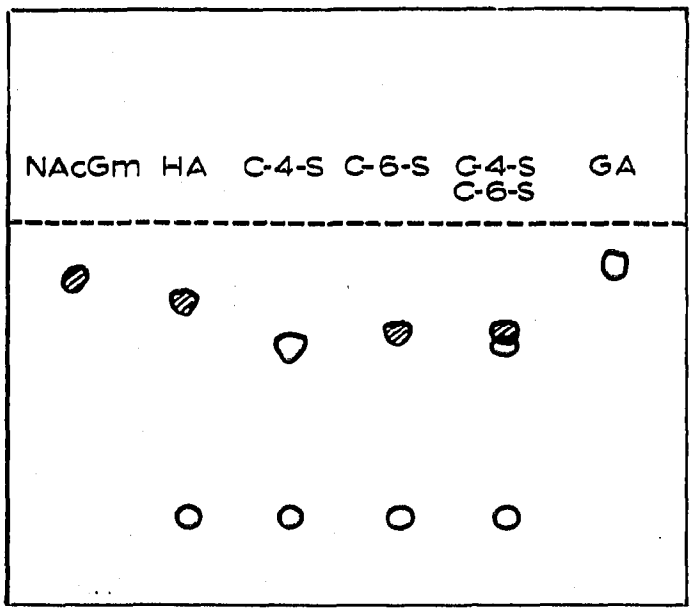

Fig. 1. Detection of MPS by chromatography of hyaluronidase digest on plates of plain silica gel. Chromatogram developed for 60 min with 2-propanol–acetic acid–water (2:1:1, v/v) and sprayed with naphthoresorcinol reagent. Control spots of N-acetyl D-glucosamine (NAcGm, yellow spot) and D-glucuronic acid (GA, blue spot). Spots also detectable by purple colour with Morgan-Elson spray indicated by hatched areas.

Since testicular hyaluronidase produces hybrid oligosaccharides by transglycosylation when acting on mixed substrates[11], care has to be exercised when interpreting the chromatographic pattern when major quantities of more than one digestible polymer are seen to be present. Synthetic mixtures containing equal proportions of hyaluronate, chondroitin 4-sulphate, and chondroitin 6-sulphate produced the three expected spots together with badly defined smears of intermediate R_F which were attributed to small quantities of hybrid tetrasaccharide.

Detection of heparin and heparan sulphate

Of the MPS listed here only heparin and heparan sulphate are degraded by

treatment with nitrous acid. They are resolved as intact polymers by an adaptation of the paper chromatographic system of CASTOR AND DORSTEWICZ[3] and detected by the specific nitrous acid–indole spray (Fig. 2a). It should be noted that resolutions of MPS as complexes with CPC depend on the relative anionic character of the polymers. Selective removal of the N-sulphate groups of heparin (by mild acid hydrolysis) yields a product which has the same chromatographic mobility as heparan sulphate. Thus partial desulphation of heparin during over-rigorous isolation procedures can lead to an artefact indistinguishable from heparan sulphate.

Detection of keratan sulphate and dermatan sulphate

Keratan sulphate is distinguished from other MPS by its greater mobility in ethanol–triethylamine–water mixture either before (Fig. 2b) or after (Fig. 3) degradation of those MPS which are susceptible to attack by testicular hyaluronidase or

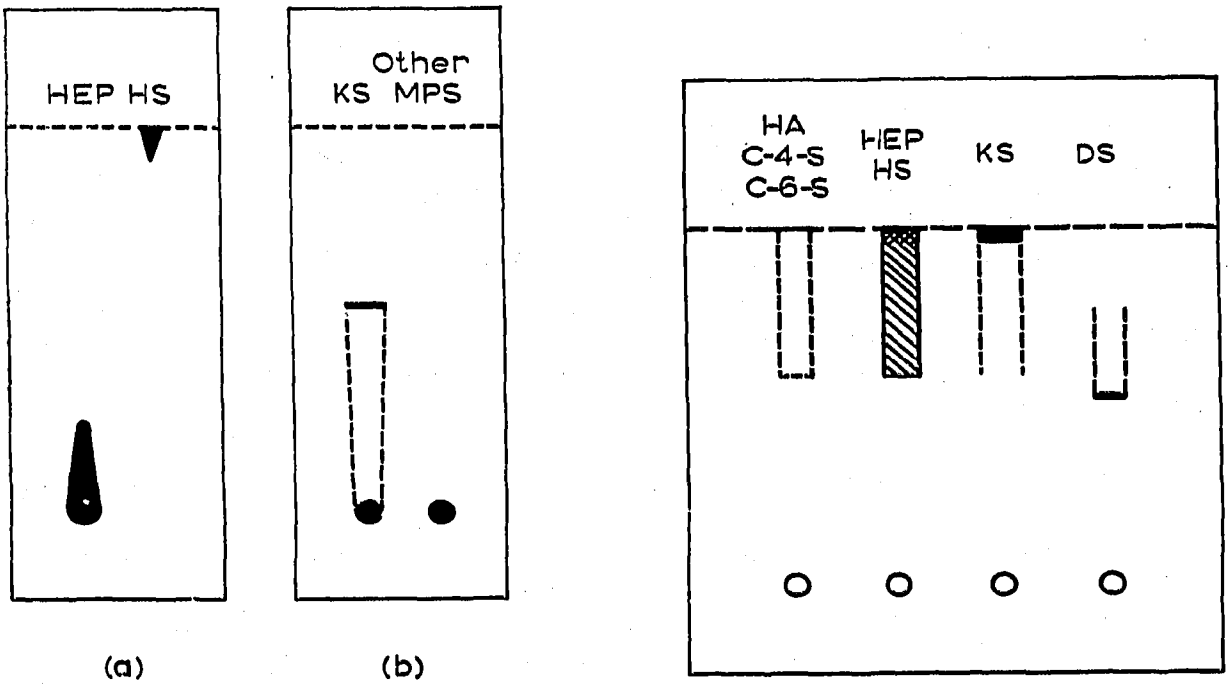

Fig. 2. Detection of single MPS in mixtures by direct chromatography. (a) Plate of silica gel impregnated with CPC developed for 30 min with 0.5 *M* magnesium chloride and sprayed with nitrous acid–indole reagent. Detection of heparin and heparan sulphate. (b) Plate of plain silica gel developed for 60 min with ethanol–triethylamine–water (30:2:5, v/v) and sprayed with orcinol reagent. Detection of keratan sulphate.

Fig. 3. Chromatography of MPS mixtures after treatment first with testicular hyaluronidase then with nitrous acid. Plate of plain silica gel developed for 60 min with ethanol–triethylamine–water (30:2:15, v/v) and sprayed with orcinol reagent. Those spots also rendered visible by indole reagent (hatched areas) or metachromatic stain (solid areas) also shown.

nitrous acid. It is located by the orcinol spray. Dermatan sulphate cannot readily be resolved from the other chondroitin sulphates, heparin or heparan sulphate. The latter are therefore degraded by hyaluronidase or nitrous acid leaving dermatan sulphate as the only metrachromatically-staining spot on the chromatogram developed as described in Fig. 3.

Proposed scheme for qualitative analysis of mixtures of MPS

Though no one chromatographic system is capable of resolving the seven common MPS in a way which allows each to be identified rapidly, the techniques described in the present paper can be combined in a simple scheme for the qualitative analysis of a mixture of as many as all seven of the polymers. In principle the polysaccharides are divided into three categories:

(A) Those susceptible to testicular hyaluronidase. After overnight digestion of a portion of the solution, aliquots are examined by chromatography as described in Fig. 1.

(B) Those degraded by nitrous acid. After further treatment of the hyaluronidase digest with nitrous acid, aliquots of the solution are chromatographed as in Fig. 3. Fragments from heparin or heparan sulphate can be detected, but not distinguished, by means of the indole spray. The two polymers are distinguished as undegraded molecules by chromatography as described in Fig. 2a.

(C) Those unattacked by hyaluronidase or nitrous acid. Duplicate runs with different sprays as in Fig. 3 permit detection of keratan sulphate and dermatan sulphate. The presence of the former can be confirmed by chromatography of the undegraded mixture as described in Fig. 2b.

This scheme has been tested exhaustively on 100 μl portions of aqueous solution containing 0.5–1.0 mg of mixed polysaccharide. Since each layer spot requires no more than 2 μl of solution only manipulative difficulties prevent the scale of the tests from being reduced to where less than 100 μg of mixed polysaccharide is required.

Pretreatment of crude polysaccharide mixture

Covalently-bound protein should be reduced to minimal levels even though it has not been found to interfere significantly when present in proportions as high as 20% by weight of the MPS mixture. Proteolytic digestion or treatment with dilute alkali are commonly used for this purpose. Since it is possible that polysaccharide chains of more than one type are linked through a peptide chain which is resistant to one of these treatments it is preferable that both be used in sequence. The product should be checked for the presence of nucleic acid (which obscures the colour reactions for keratan and dermatan sulphates), oligosaccharides (which interfere with the examination of the products of hyaluronidase digestion) and free hexosamine (which gives a positive nitrous acid–indole reaction).

DISCUSSION

Previous reports of chromatographic schemes to identify unknown MPS on glass paper or ordinary filter paper emphasise the difficulties presented by this group of polysaccharides. Polydispersity, chemical heterogeneity, and attachment of varying quantities of peptide all make it difficult to identify one member of the group in the presence of others, particularly by the normal chromatographic techniques all of which are very sensitive to such factors. In common with the schemes of BERENSON AND DALFERES[2] and CASTOR AND DORSTEWICZ[3] the system of analysis described here requires several independent chromatographic steps. Through the use of thin layers, however, the quantities of material required for detection and the time taken to perform the runs are reduced considerably. The use of specific degradation

procedures and spray reagents make it unnecessary to use standard polysaccharide preparations as controls once the materials used have been checked by trial runs. The two MPS available commercially in a purified state (heparin and hyaluronate) are quite adequate for this purpose.

SUMMARY

Thin-layer studies on mixtures of acidic glycosaminoglycans and the products of digestion with testicular hyaluronidase are described. Specific colour reactions for the polysaccharides and related oligosaccharides are described and a scheme proposed for the identification of the components in a mixture of acidic glycosaminoglycans.

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