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Gas-chromatographic characterization of the electrophoretically separated fractions of acid mucopolysaccharides

Acid mucopolysaccharides can be separated by elution of columns of cellulose[1] (after precipitation with cetylpyridinium chloride), Dowex-1[2] or DEAE-Sephadex[3] with magnesium chloride or sodium chloride solutions of increasing concentration. Relatively large (about 10 mg) quantities of mucopolysaccharides are required for these methods.

Very small (5–10 μg) samples of mucopolysaccharides can be analyzed by means of cellulose acetate electrophoresis[4,5], but it has been difficult to characterize the fractions chemically.

Materials and methods

Materials. D-Glucosamine and D-galactosamine, homogenous in paper chromatography, were obtained from Mann Research Laboratories Inc., New York, N.Y., D(+)-glucose, analytical reagent grade, from B.D.H., Poole, Great Britain; galactose, *puriss.* from E. Merck A.G., Darmstadt, Germany; D(+)-glucuronic acid lactone, *puriss.* from Fluka AG, Buchs, S.G., Switzerland; hexamethyldisilazane, *purum* 98 %, and trimethylchlorosilane, *puriss.* 99 %, from Fluka AG, Buchs, S.G., Switzerland; pyridine, reagent grade, redistilled, anhydrous, J. T. Baker Chem. Co., Phillipsburg, N.J.; cetylpyridinium chloride, Recip AB, Stockholm, Sweden; oxoid electrophoretic strips, The Oxoid Division, OXO Ltd., London; Dowex-50, J. T. Baker Chem. Co., Phillipsburg, N.J.; and alcian blue, Gurr Ltd., London.

Iduronic acid was prepared from chondroitin sulphate B and was a gift from K. VON BERLEPSCH, F. Hoffman-La Roche Ltd., Basel, Switzerland.

Samples of keratosulphate and heparitin sulphate were obtained from Dr. M. B. MATHEWS, University of Chicago, Chicago, Ill., and from Prof. K. MEYER, Columbia University College of Physicians and Surgeons, New York, N.Y.

Extraction of mucopolysaccharides. The acid mucopolysaccharides were liber-

ated from acetone-defatted bovine skin by hydrolyzing with papain[2]. Trichloroacetic acid (10 % w/v) was added to precipitate the proteins and nucleic acids, and it was removed from the supernatant with ether. The acid mucopolysaccharides were precipitated at 4° with 4 volumes of ethanol which contained 0.5 % of sodium acetate and the precipitate dissolved in water. The sample was further purified by a precipitation with an excess cetylpyridinium chloride (CPC). The CPC-complexes were dissolved in 3 N $MgCl_2$ and the acid mucopolysaccharides reprecipitated with ethanol.

Electrophoretic fractionation. Electrophoresis of mucopolysaccharides was performed on Oxoid cellulose acetate sheets, using a barbiturate buffer, pH 8.6[4], voltage gradient 15 V/cm, and a running time of 25 min. A small strip was cut off the sheet and stained with alcian blue (1 % solution w/v in 25 % acetic acid). The bands were then cut off the unstained parts of the sheets and eluted with water.

Gas chromatography. The carbohydrate components were analyzed as their trimethylsilyl derivates[6–7]. For the determination of hexosamines the mucopolysaccharide fractions were hydrolyzed in 1.5 N HCl in sealed tubes at 103° for 17 h. The separation of hexosamines from other carbohydrates was accomplished by using columns of Dowex-50[8]. Uronic acids and neutral sugars were analyzed after hydrolysis of the fractions in 1 N HCl at 100° for 3 h. The amino sugars were removed from the hydrolyzates with Dowex-50.

The hydrolyzates were evaporated to dryness, pyridine (0.175 ml), hexamethyldisilazane (0.05 ml) and trimethylchlorosilane (0.015 ml) were added successively. The mixture was shaken and allowed to stand for 30 min at room temperature and the excess of reagents were evaporated off in a stream of nitrogen. The trimethylsilyl ethers were extracted with 5 ml of *n*-hexane and concentrated to the desired volume.

A Barber Colman M-10 chromatograph equipped with a flame ionization detector was employed. The column conditions were as follows: 6 ft. × 3 mm, 1 % SE-30 on 100–140 mesh siliconized Gas-chrom P (Applied Science Laboratories Inc.). Temperature 140°. Carrier gas was nitrogen with a flow rate of 30 c.c./min, pressure 0.5 atm.

Results and discussion

The mucopolysaccharides of the bovine skin were electrophoretically separated nto three fractions (Fig. 1). The electrophoretic mobility of fraction 1 corresponded

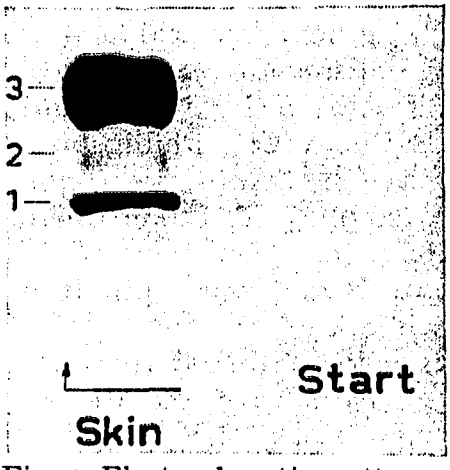

Fig. 1. Electrophoretic pattern of acid mucopolysaccharides of bovine skin. Sample size 10 μg.

to hyaluronic acid which had been isolated from human umbilical cord. Fraction 2 moved in the same manner as the reference samples of heparitin sulphate or keratosulphate and fraction 3 as chondroitin sulphate B. The gas chromatographic analyses showed that galactosamine and a small amount of glucosamine were present in fraction 3 and glucosamine only in fractions 1 and 2 (Fig. 2). The determination of uronic acids and neutral sugars showed that iduronic acid was present in fraction 3, galactose in fraction 2, and glucuronic acid in fraction 1 and also in fraction 3 as a minor component (Fig. 3). When pure cellulose acetate sheets were eluted with water, some glucose was observed in the eluate and it was present also in all the mucopolysaccharide fractions. The quantity of sugars was large enough for identification in samples pooled from 4–8 electrophoretic sheets (total mucopolysaccharides 40–100 μg). After hydrolysis for 3 h small unidentified peaks, possibly impurities, were observed in the gas chromatographic patterns of fractions 1 and 3.

On the basis of this semiquantitative determination of the carbohydrate components, fraction 1 was hyaluronic acid, fraction 2 keratosulphate, and fraction 3 contained chondroitin sulphates, mainly chondroitin sulphate B.

The electrophoresis of mucopolysaccharides on cellulose acetate sheet is a rapid method for the fractionation of small mucopolysaccharide samples. The electrophoretic mobility of keratosulphate and heparitin sulphate is the same at pH 8.6,

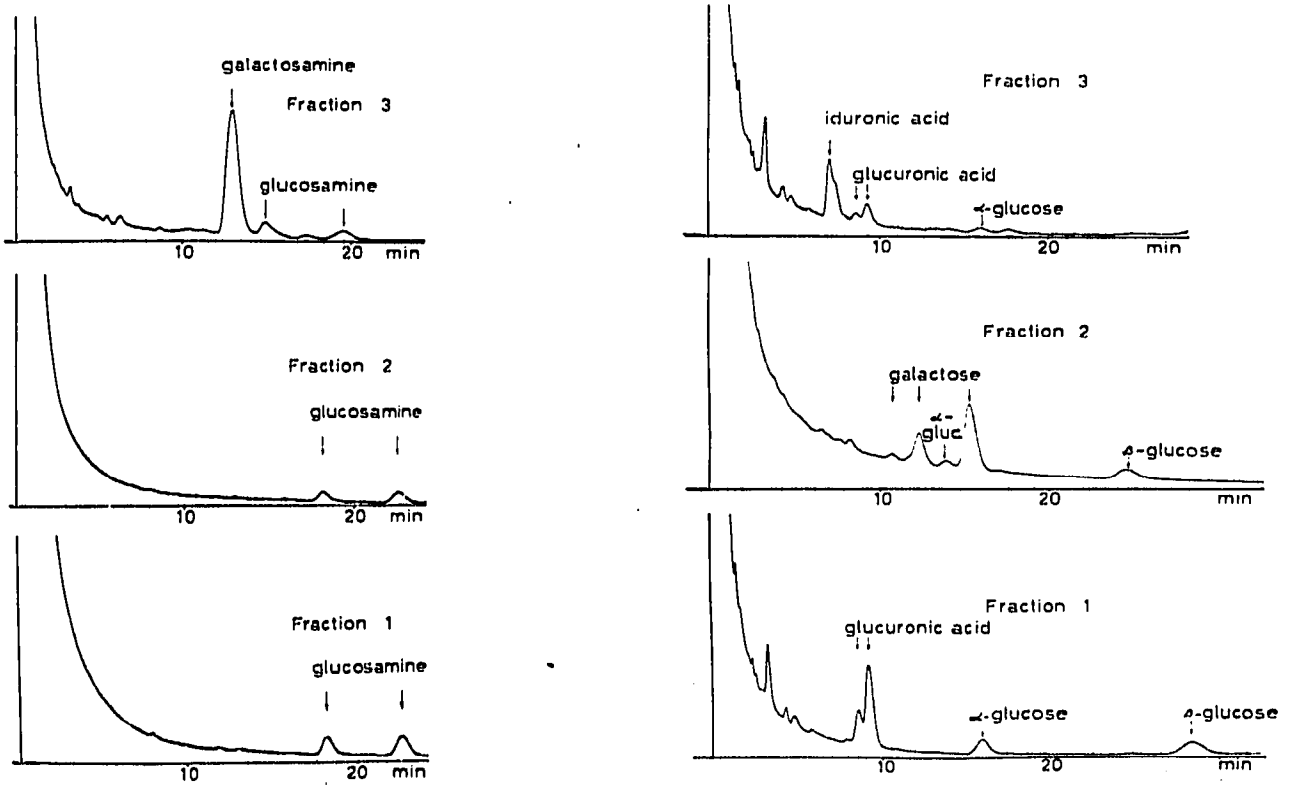

Fig. 2. Gas chromatography of amino sugars of electrophoretically separated mucopolysaccharide fractions. Fraction numbers 1–3 refer to those in Fig. 1. Sample obtained by eluting 4 electrophoresis strips, corresponding to 40 μg total mucopolysaccharide.

Fig. 3. Gas chromatography of uronic acids and neutral sugars of electrophoretically separated mucopolysaccharide fractions. Fraction numbers 1–3 refer to those in Fig. 1. Sample obtained by eluting 8 electrophoresis strips, corresponding to 80 μg total mucopolysaccharide.

but heparitin sulphate and keratosulphate can be differentiated by analysis of the carbohydrate components.

The disadvantage of this method, however, is that chondroitin sulphates A, B and C cannot be separated from each other; though iduronic acid of chondroitin sulphate B and glucuronic acid of chondroitin sulphate A and C can be differentiated by gas chromatography. The quantitative determination of the uronic acids by this method is under investigation.

This procedure is useful for the qualitative and semiquantitative analysis of the tissue polysaccharides, when the individual electrophoretic fractions are available in amounts of 5–50 μg.

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Quantitative determination of griseofulvin by gas–liquid chromatography

Several methods have been described for the determination of griseofulvin in many types of mixtures. The use of a spectrophotometric method[1] is relatively non-specific and therefore an indirect method[2], based on the conversion of griseofulvin to isogriseofulvin, was devised for quantitating griseofulvin in the presence of structurally similar contaminants. There is a spectrophotofluorometric assay[3,4] which is rather more specific and has been used extensively for detecting and estimating griseofulvin in biological fluids. In this assay, however, aspirin, salicylic acid and quinine are likely to interfere[5]. Recently a liquid–solid chromatographic method[6], although the procedure is tedious and time consuming, has been reported for the direct analysis of griseofulvin in complex fermenter broths.

In this study, a new gas chromatographic method was successfully developed for