CHROM. 6157

Thin-layer chromatographic separation of glycosaminoglycans

The similarities in their chemical structures and the wide variation in their molecular weights makes the separation of glycosaminoglycans (GAG) by various chromatographic and electrophoretic methods difficult[1]. Numerous attempts have been made to separate and identify GAG from both tissue extracts and biological fluids by these methods. Electrophoretic studies include those by CLAUSEN AND ASBOE-HANSEN[2] with paper, and by MANLEY AND HAWKSWORTH[3] and ONISAWA AND LEE[4] with cellulose acetate. Paper chromatographic methods were applied by BERENSON AND DALFERS[5], CASTOR AND DORSTEWICZ[6] and WALLACE *et al.*[7]. Thin-layer (TLC) chromatographic methods have been reported by WOSTEMANN *et al.*[8], BISCHEL *et al.*[9], MARZULLO AND LASH[10], TELLER AND ZIEMANN[11], HUMBEL *et al.*[12] and recently by LIPPIELLO[13]. In some of the TLC methods[8,9], digestion with hyaluronidase or degradation with nitric acid were used, but the chromatograms thus developed were not satisfactory. TELLER AND ZIEMANN[11] and HUMBEL *et al.*[12], who chromatographed GAG obtained from urine, obtained better R_F values. Good results were obtained when the chromatogram was developed in sections on a layer impregnated with the silicate of LIPPIELLO. In the present paper, we report the results obtained during the TLC separation of standard GAG.

The reference GAG standards

The reference GAG standards were obtained from M. B. Mathews (University of Chicago); they isolated chondroitin 6-sulphate (C-6-S) from the human umbilical cord, chondroitin 4-sulphate (C-4-S) from the rock sturgeon *(Acipenser fulvescense)*, dermatan sulphate (DS) from the mucous membrane of the pig, keratan sulphate (KS-2) from human costal cartilage, heparin (He) from the mucous membrane of the pig and heparan sulphate (HS) from bovine lung. Aqueous solutions of concentration 1 mg/ml were prepared from the various GAG solutions and were stored in a deep-freezer at −20°. The standards were used for about 1 month; during this time every standard remained usable, although that of the KS-2 did deteriorate.

Preparation of thin-layer plates

Microcrystalline cellulose plates pre-coated with a layer of MN-Polygram cellulose 400 (Macherey-Nagel and Co.), 0.1-mm thick, were used. The 20 × 20 cm plates were cut up as required. Volumes of 5 μl of sample were dripped on to the base of the plates with an Eppendorf automatic micropipette. After drying the standards, the bases of the plates were impregnated to a distance of 5 cm above the starting point with the Parwaresch-modified Mota solution used to fix blood basophilic cell granules[14] (3 g of lead acetate were dissolved in 80 ml of 96% ethanol and a mixture of 20 ml of 40% formalin and 1 ml of glacial acetic acid was added to it). The impregnation lasted for 5–10 sec, and then after drying for 5–10 min at room temperature the chromatogram was developed. The impregnation solution was always kept at +4°and before use it was always necessary to heat it to room temperature so as to dissolve the crystallized lead acetate.

Solvent systems

Two systems were used for the separation of the GAG:

(1) For the separation of GAG in urine, HUMBEL *et al.*[12] used 0.04 *M* ammonium formate–methanol (45:55), containing 1.1 m*M* complexone-III.

(2) 0.04 *M* ammonium formate–isopropanol (50:50), containing 1.1 m*M* complexone-III.

From the point of view of reproducibility, it is very important that the 0.04 *M* ammonium formate of pH 4.3 be accurately prepared. As it was observed that the quality of the chromatogram was seriously impaired by the non-saturation of the running-baths, they were saturated for 72 h before use with the vapour of the running-agent. In all instances the run was carried out room temperature.

Detection

After the development of the chromatograms, the plates were dried and treated with a dye solution[12] consisting of 50 mg of Azure A in a mixture of 20 ml of acetone,

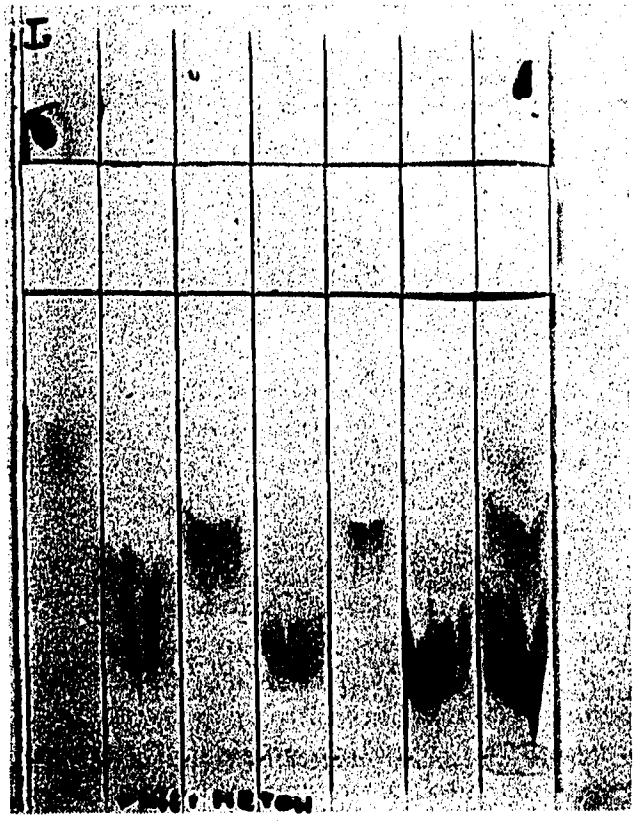

Fig. 1. Chromatograms of the six GAG in system 1. Order of runs from left to right: KS-2 HS, C-4-S, DS, C-6-S, He and a mixture of the six GAG.

60 ml of methanol and 20 ml of 2% acetic acid. After a treatment for 2–3 min, the chromatogram was washed with 1% acetic acid solution. The GAG spots appeared as purple spots on a light blue background.

Results

In system 1, a period of about 3 h was necessary for the development of the chromatogram. The six GAG separated into three spots. In the highest position, at $R_F = 0.42$, is the spot of KS-2 (with KS-2, compounds were always found at the front of the chromatogram, which were likewise metachromatically stained; it is assumed that these compounds are degradation products of the reference compound). In the middle, at $R_F = 0.31$, are C-4-S and C-6-S, while in the lowest position, from $R_F = 0.14$ to 0.22, are HS, DS and He (Fig. 1). On the right on the first band, all six GAG are combined. If the chromatogram is run for 24 h so that the running agent reaches the upper edge of the chromatogram, then the R_F values of the individual

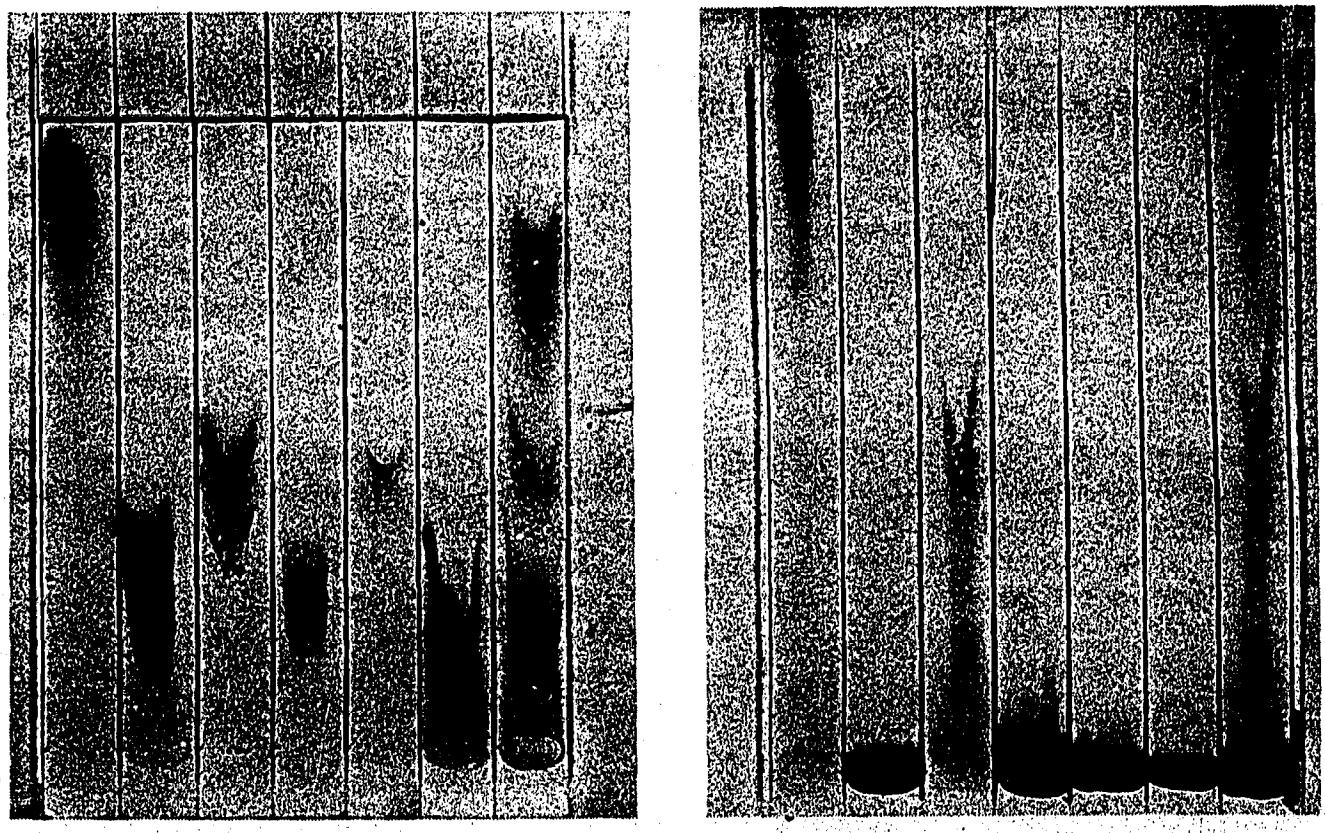

Fig. 2. Chromatograms of the six GAG in system 1, developed for 24 h. Order of runs from left to right: KS-2, HS, C-4-S, DS, C-6-S, He and a mixture of the six GAG.

Fig. 3. Chromatograms of the six GAG in system 2. Order of runs from left to right: KS-2, HS, C-4-S, DS, C-6-S, He and a mixture of the six GAG.

GAG components will be larger and so they will be better separated from each other (Fig. 2).

In system 2, C-4-S and C-6-S were separated (Fig. 3). In this system, C-6-S remains at the start, while C-4-S, with a long tail, runs together with KS-2 but with its spot below that of KS-2.

Discussion

The development of the above two systems was preceded by many preliminary experiments, during which the pH of the buffer applied and the composition of the running mixture were varied. If the amount of the ammonium formate buffer is increased and that of methanol decreased, then the R_F values of the standards increase. If the chromatogram is developed in an ammonium formate–methanol (53:45) system, the R_F values of the standard GAG are higher than those of the standards on a chromatogram developed in an ammonium formate–methanol (45:55) system.

The individually applied standards give slightly elongated spots, which at first may appear to be a difficulty in the evaluation of the chromatogram. If, however, these standards are taken on a common band, then it can be seen that the individual fractions are well separated from each other. Boric acid impregnation, known from sugar chromatography, was also tried before the application of the Mota solution.

TABLE I

R_F VALUES OF THE SIX GAG STANDARDS

GAG standard	*R_F values*		
	System 1	*System 1 (developed 24 h)*	*System 2*
KS-2	0.43	0.75	0.82
HS	0.21	0.22	0.00
C-4-S	0.31	0.36	—
DS	0.15	0.23	0.00
C-6-S	0.32	0.35	0.00
He	0.14	0.16	0.00

In system 2, on the other hand, only KS-2 and C-4-S move; KS-2 has the higher R_F value, and the C-4-S gives a less well defined, elongated spot. In contrast, C-6-S, in a similar manner to DS, HS and He, does not move from the starting point (Table I). During our investigations we first used a metal-containing solution in the chromatography of the GAG, essentially to promote and ensure the separation and reproducibility of the individual fractions, the application of which was known only in the histochemistry of GAG. As it did not prove possible to separate the free GAG in a satisfactory manner, we attempted to separate GAG in the form of metal salts. For this reason we used the Mota solution, in which lead salts of GAG are formed; if the complexes were prepared with complexone-III, a good separation resulted. The various GAG are not uniformly stained by Azure A, so that it is not possible to draw quantitative conclusions from the intensities of the colours.

Our investigations were further developed for the separation of GAG in urine, which is important in paediatric diagnosis. From this work also, it appears that C-4-S and C-6-S excreted in normal urine separate from those pathological GAG fractions that are excreted in larger amounts only by patients suffering from mucopolysaccharidosis syndrome. We should like to develop our method further with the aim of establishing a rapid qualitative routine investigation that could possibly be used as a screening test.

We are indebted to Dr. MARTIN B. MATHEWS of the University of Chicago, who kindly provided the standards.

Pediatric Department,
University of Szeged,
Szeged (Hungary)

ZOLTÁN HAVASS
LAJOS SZABÓ

1 S. GARDELL, *Biochemical Society Symposium No. 20: The Biochemistry of Mucopolysaccharides of Connective Tissue*, Cambridge University Press, London, 1961, p. 39.
2 J. CLAUSEN AND G. ASBOE-HANSEN, *Clin. Chim. Acta*, 13 (1966) 475.
3 G. MANLEY AND J. HAWKSWORTH, *Arch. Dis. Child.*, 41 (1966) 91.
4 J. ONISAWA AND T. Y. LEE, *Biochim. Biophys. Acta*, 208 (1970) 144.
5 G. S. BERENSON AND E. R. DALFERS, *Biochim. Biophys. Acta*, 58 (1962) 34.
6 C. W. CASTOR AND E. R. DORSTEWICZ, *J. Chromatogr.*, 13 (1964) 157.
7 B. J. WALLACE, D. KAPLAN, M. ADACHI, L. SCHNECK AND B. W. VOLK, *Arch. Pathol.*, 82 (1966) 462.
8 F. S. WUSTEMAN, A. G. LLOYD AND K. S. DODSIN, *J. Chromatogr.*, 21 (1966) 32.
9 M. D. BISCHEL, J. A. AUSTIN, M. D. KEMÉNY, C. M. HUBBLE AND R. K. LEAR, *J. Chromatogr.*, 21 (1966) 40.
10 G. MARZULLO AND J. W. LASH, *Anal. Biochem.*, 18 (1967) 575.
11 W. M. TELLER AND A. ZIEMANN, *Horm. Metab. Res.*, 1 (1969) 32.
12 R. HUMBEL, C. MARCHAL AND M. FALL, *Helv. Pediatr. Acta*, 24 (1969) 648.
13 L. LIPPIELLO, *Anal. Biochem.*, 39 (1971) 54.
14 M. R. PARWARESCH AND K. LENNET, *Z. Zellforsch.*, 83 (1967) 279.

Received March 30th, 1972