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TWO-DIMENSIONAL ELECTROPHORESIS OF URINARY MUCOPOLYSACCHARIDES ON CELLULOSE ACETATE AFTER *N*-CETYLPYRIDINIUMCHLORIDE (CPC) PRECIPITATION: A METHOD SUITABLE FOR THE ROUTINE LABORATORY

N.G.G.M. ABELING[a], S.K. WADMAN[a,*] and A.H. VAN GENNIP[b]

[a] *Wilhelmina Children's Hospital of the University of Utrecht, Nieuwe Gracht 137, Utrecht, and* [b] *Emma Children's Hospital, Spinozastraat 51, Amsterdam (The Netherlands)*

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Summary

A technique for two-dimensional electrophoresis of urinary mucopolysaccharides (MPS) is described. The method allows differentiation of a number of mucopolysaccharidoses and is suitable for application in the routine laboratory.

This technique should be used to evaluate urines from patients who have a positive MPS-spot test and/or an increased urinary excretion of MPS-bound uronic acid. Urinary MPS excretion patterns are given from normal individuals, patients with the Sanfilippo syndrome and the Hunter syndrome.

Introduction

The determination of the urinary excretion pattern of the mucopolysaccharides (MPS) is of great value for the diagnosis of the mucopolysaccharidoses (MPSoses). The methods currently used for this purpose are precipitation followed by column fractionation and quantitative analysis of each fraction [1—3]. A more convenient method is one-dimensional electrophoresis after isolation and concentration of the MPS [4]. Although this method is more rapid, the resolution is incomplete and the fractions cannot be easily recognized.

The advantages of the two-dimensional method compared to column chromatography are rapidity, better resolution and higher sensitivity. Compared with one-dimensional electrophoresis the striking advantage of the two-dimensional method is the easy, semi-quantitative recognition of the excretion patterns. Moreover, keratan sulphate, a component very difficult to determine in the methods mentioned above, is relatively well separated by two-dimensional electrophoresis, as is heparin.

* To whom requests for reprints should be addressed.

The method described in this paper is a modification of procedures proposed by Hata and Nagai in 1972 [5] and Whiteman in 1973 [6], who introduced his technique for clinical diagnosis. Electropherograms are included from normal and pathological urines. We recommend this method as suitable for the routine laboratory and hope it will stimulate interest in the analysis of urinary mucopolysaccharides in clinical chemistry.

Materials

Cellulose acetate strips (17 cm × 17 cm, Sepraphore III. Gelman Instrument Co., Ann Arbor, Michigan).

Electrophoresis chamber (24 cm × 31.2 cm × 5 cm, Gelman Instrument Co., Ann Arbor, Michigan).

Buffer systems: in the first direction, pyridine—acetic acid—water (100 : 10 : 890, v/v/v), pH 6.0; in the second direction, 0.1 M barium acetate in water, pH 8.3.

Alcian Blue 8Gx/8GS, p.a. Baker, 0.25% in methanol—acetic acid—water (50 : 5 : 45, v/v/v).

N-Cetylpyridiniumchloride (CPC), p.a. Merck, 5% in water.

Spot test: MPS TM Papers, Ames-Yissum Ltd, Jerusalem, Israel.

Standards: chondroitin 4-sulphate (C4S, prepared from whale cartilage), chondroitin 6-sulphate (C6S, prepared from shark cartilage) and dermatan sulphate (DS), were obtained from Seikagaku Kogyo Co. (Tokyo, Japan); hyaluronic acid from Miles Laboratories, Elkhart, U.S.A.).

Urine samples were collected on ice and frozen immediately.

Methods

Precipitation

Precipitation is mainly according to Di Ferrante and Rich [2] and Giesberts et al. [3]. A 25 ml well-mixed 24 h aliquot of a urine sample is centrifuged at 10 000 rpm for 10 min and the precipitate discarded. Twenty ml of the supernatant is taken for further analysis. Fifteen ml of water is added to the aliquot and the pH brought to 4—5 with 1 *N* HCl. Two ml 5% CPC is added, the mixture stirred vigorously and allowed to equilibrate for 12 h at 4°. The complex formed is separated from the mixture by centrifugation (10 min at 10 000 rpm and 4°), resuspended in a few millilitres of ethanol saturated with NaCl and centrifuged (10 min at 10 000 rpm and 4°) (×2). The complex is dried by heating the tube in a boiling waterbath and then dissolved in 2 ml 0.05 *N* NaOH. This solution is used as an extract for electrophoresis.

Electrophoresis

Before analysis, a Sepraphore III cellulose acetate strip (8.5 cm × 8.5 cm) is saturated with buffer for a couple of hours. The strip is placed on a glass plate and lightly blotted to remove excess moisture; air bubbles should be avoided between the strip and glass plate. A capillary tube is used to apply 10 µl of the MPS extract (see above) to the corner of the strip, at a distance of 1 cm from each side. The glass plate is then fixed in the electrophoresis tank

and the strip connected with the buffer solution by means of filter paper strips. The system should be allowed to equilibrate for 5 min before each run.

First run. 1 h 20 min at 10V/cm in pyridine—acetic acid—water (100 : 10 : 890, v/v/v), pH 6.0. After this run the strip is allowed to dry for 30 min in air. Before the second run the strip is carefully moistened by laying it gradually onto the surface of 0.1 M barium acetate solution for only a few seconds. It is then quickly placed on a filter paper to remove excess solution and replaced on the glass plate in a position at a right angle to the first run.

Second run. 3 h at 10V/cm in 0.1 M barium acetate pH 8.3. Immediately after the run the strip is stained for 15 min in 0.25% Alcian Blue solution (solvent is methanol—acetic acid—water (50 : 5 : 45, v/v/v)), washed three times in 5% acetic acid for about 15 min until the blue background has fainted, and finally cleared for 10 min in a solution of dimethylformamide—acetic acid—water (30 : 15 : 55, v/v/v), The strip is then placed carefully (avoiding air bubbles) on a glass plate; excess clearing solution is taken away very carefully with filter paper and the glass plate placed quickly in an oven at 100°. After ± 10 min the strip is clear and the colour fixed.

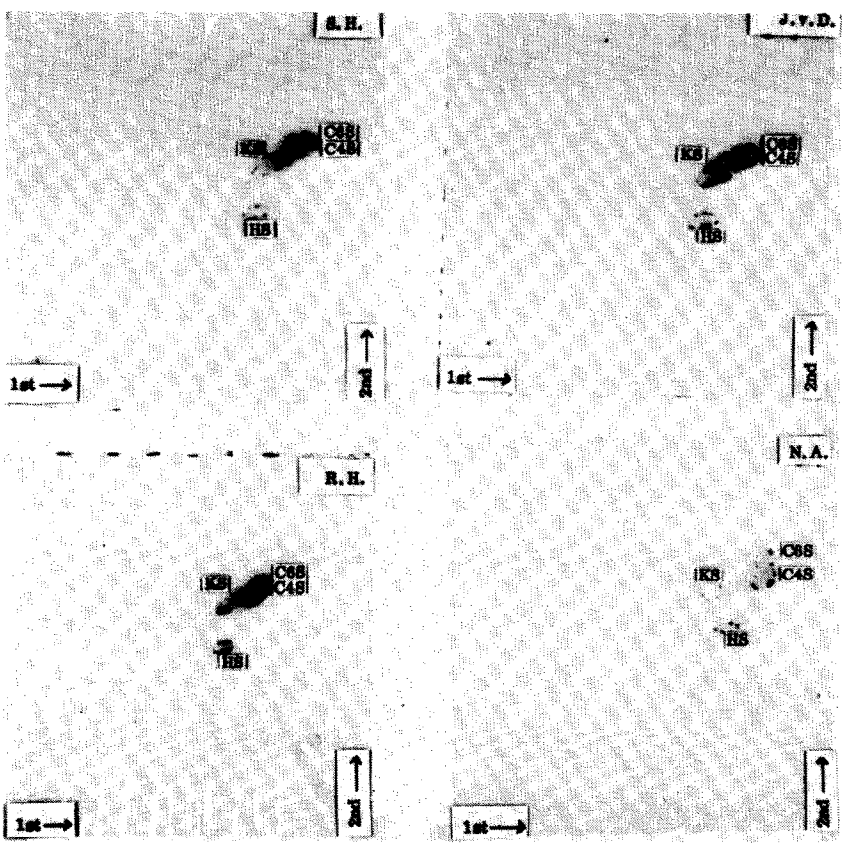

Fig. 1. Electropherograms showing normal pattern of urinary MPS. In R.H. total MPS excretion, expressed as uronic acid, was increased but the pattern was normal. Spots of C4S + C6S. KS and HS can be distinguished.

Results

Abbreviations of the patients' names and of the various MPS are indicated in the figures. The arrows indicating the first and second directions of migration point towards anode.

Fig. 1 shows the electropherograms of three subjects, S.H., J.v.D. and N.A., with normal MPS excretion expressed as uronic acid. The main mucopolysaccharides present are chondroitin 4-sulphate (C4S), chondroitin 6-sulphate (C6S) (not separated) and keratan sulphate (KS). A trace of heparan sulphate (HS) can also be seen. Since KS and HS are not commercially available their identification is tentative and based upon their relative positions in the standard electropherograms of Hata and Nagai [5]. In one subject, R.H. (a 16-year-old blind psychomotorically retarded boy) a normal pattern was observed but the MPS excretion expressed as uronic acid was clearly elevated (19 mg/24 h).

Fig. 2 shows the electropherograms of three patients with the Sanfilippo syndrome. Intense HS and HP spots can be seen in all cases. The normal spots of C4S + C6S and KS are present as well. A trace of dermatan sulphate (DS) is also observed.

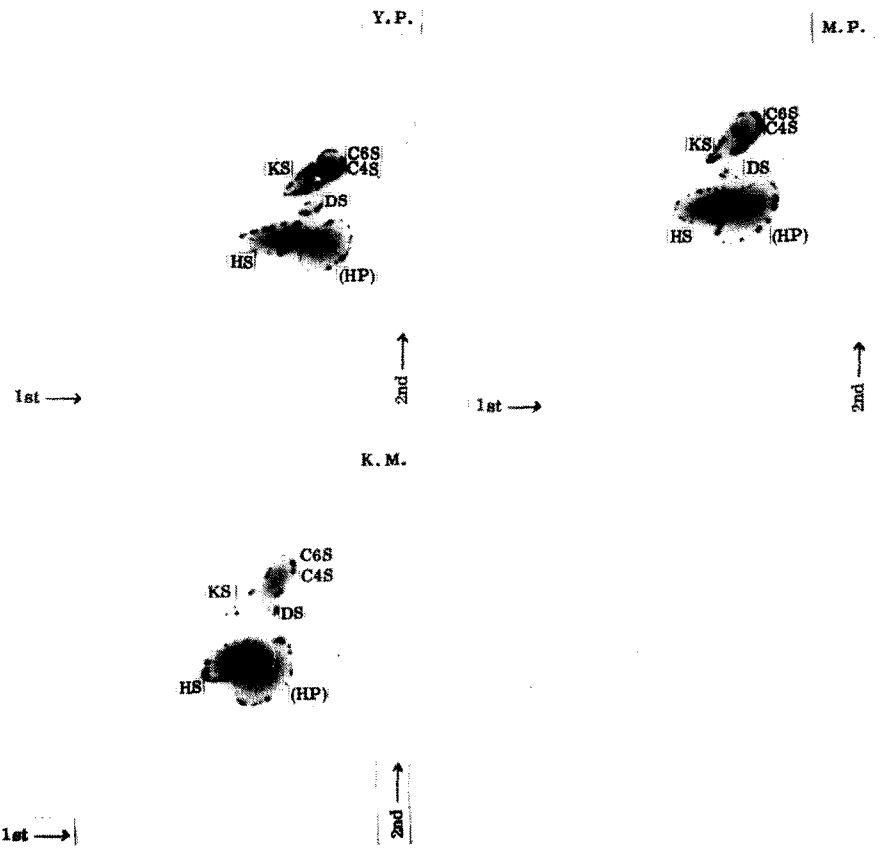

Fig. 2. Electropherograms showing patterns of urinary MPS in 3 patients with the Sanfilippo syndrome. Strong spots of HS and HP are observed.

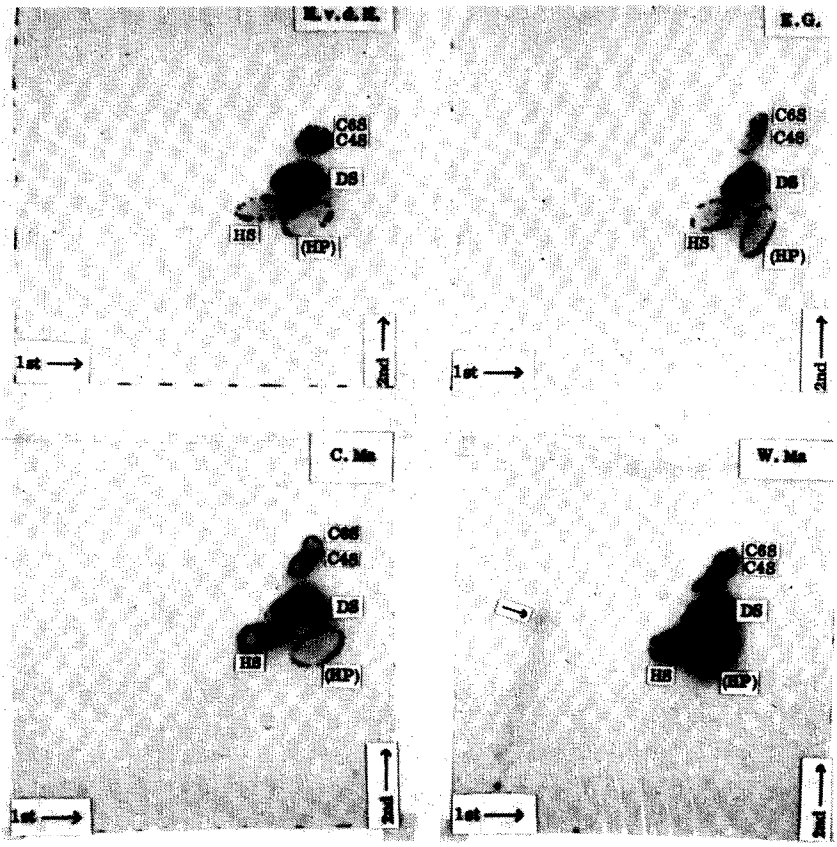

Fig. 3. Electropherograms showing patterns of urinary MPS in 4 patients with the Hunter syndrome. A very strong DS spot is observed, also HS and HP seem to be increased. An unidentified tailing compound was present (indicated by arrow) in patient W.Ma.

The electropherograms of four patients with the Hunter syndrome are presented in Fig. 3. The most striking feature of these patterns is the strongly coloured DS spot. Relatively strong spots of HS and HP can also be seen; C6S or C4S are also present. However, KS seems to be absent in the pattern. One subject (W.Ma.), has a weak, unidentified tailing spot of an apparently low-sulphated compound.

Discussion

The separation of compounds is based upon sulphate content + polysaccharide structure. The sulphate content is primarily responsible for the mobility of the MPS in the first direction at pH 6. The migration in the second direction at pH 8.3 is determined by the polysaccharide "backbone" structure. It should be emphasized that this method is not complete in the sense that it gives only the distribution of the CPC-precipitable urinary MPS, the large molecular weight part. This part varies from 20—40% in normal to 50—70% in pathological urines [4]. According to Wessler [10], the large molecular weight

part of the urinary MPS of normal subjects (determined in this case as not-ultrafilterable material) consists grossly of C6S + C4S (~ 60%), KS (up to 18%) and HS (up to 15%); normally the other MPS are in the order of 1—2% or less. These figures confirm, at least semi-quantitatively, the pattern seen on the normal electropherograms given in Fig. 1.

The patterns observed for the Sanfilippo syndrome (Fig. 2) and the Hunter syndrome (Fig. 3) also agree with the elevated HS excretion and the high excretion of DS together with HS respectively. In both diseases another compound, probably HP, is consistently observed as a diffuse spot. The presence of HP has been described in patients with the Sanfilippo syndrome [11,12]. It is not easy to "prove" the identity of the compounds that can be distinguished; there are, however, indirect ways for tentative identification of the individual spots. Firstly, Hata and Nagai [5] have accomplished the two-dimensional electrophoretic separation on cellulose acetate under comparable circumstances with aqueous solutions of seven standard MPS compounds. The relative positions of the spots obtained by these workers can be used as a guide for identification of the spots observed in the patterns shown in the figures. In addition, we have done one-dimensional electrophoresis in 0.1 M barium acetate with our own standards (see Materials) to find the relative positions of C6S, C4S, DS and HP in the second direction. We could not use these standards for identification in the first direction because the sulphate contents of C6S, C4S and DS were too similar to get any separation; moreover, we lacked standards of HS and KS.

Finally, we could interpret the patterns because of the known characteristic excretions of specific MPS in the urines of previously diagnosed typical patients.

An important aspect of this method is that the heterogeneous KS does not disturb the electrophoretic pattern, as it does in one-dimensional analysis, moreover, it is reasonably separated from the other components. An overlap still exists with C6S and C4S. There are several reasons for this: the first one is the heterogeneity in sulphate content of KS causing overlap with chondroitin sulphates of the same degree of sulphation. Moreover, some workers [7—9] have isolated from urine compounds called "mixed peptidoglycans", in which chondroitin sulphates and keratan sulphate are covalently bound to the same peptide.

So far there is no buffer known to separate the very similar C4S and C6S completely. An aqueous solution of 0.1 M cupric acetate gives better results than the barium acetate used in our method but then the more important DS is badly separated from the other chondroitin sulphates [5].

The "overlap region" is essential for the detection of the Morquio syndrome, which is characterized by an elevated excretion of KS. However, the quantitative assay of the total MPS excretion by means of uronic acid determination shows no raised values in most of these cases, which is understandable as KS contains no uronic acid. But a positive spot test with Azure blue can be expected because this stain interacts with KS too. On the electropherogram, however, it may be more difficult to estimate, semi-quantitatively, the elongated, partly separated spot of KS. Especially for this diagnosis clinical data are of utmost importance. In patients with the Morquio—Ullrich syndrome both

KS and a chondroitin sulphate are excreted in excess. We could not assess whether the electrophoretic pattern is sufficiently characteristic, because no urine sample was available to us. A spot test gives positive results as does the uronic acid determination. Moreover, the clinical picture of these patients is usually very clear and characteristic. In the electrophoretic assay the same problem may arise with respect to the semi-quantitative estimation of the compounds in the "overlap region".

Finally the question arises as to when the described technique should be used in practice. The method is too elaborate to be used for a general screening; a preselection should be made on the basis of clinical data together with values obtained from spot test and uronic acid determination.

The electrophoretic results should serve as a final selection before the difficult and time consuming determination of the specific enzyme deficiency in cultured fibroblasts to confirm the definite diagnosis. From the two-dimensional patterns diagnoses can be made probable or excluded and a guide for definite enzyme assays is obtained.

The special advantage of the two-dimensional method is the possibility of pattern recognition, a property which is not a feature of one-dimensional techniques. Whiteman [6] pointed out the importance of the two-dimensional electrophoresis for analysis of MPS in amniotic fluid in prenatal diagnosis. For this purpose the precipitation step has to be scaled down.

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