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Note

Urinary glycosaminoglycans and the direct quantification of irregular spots on thin-layer chromatograms

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A variety of methods have been used for the detection of excess urinary glycosaminoglycans (acid mucopolysaccharides, AMPS) in Hurler's disease and other mucopolysaccharidoses. These techniques have made use of the interaction of these acidic macromolecules with different organic cations to give detectable products such as a metachromatic dye complex[1], an insoluble precipitate[2] or a turbid colloidal suspension[3]. In general, these techniques have suffered from poor reliability and a consequent multiplicity of improvements and modifications[4,5]. Thin-layer chromatography (TLC) provides a rapid means for distinguishing between AMPS and mucoproteins which can give a false positive spot test[6]. Partial purification of the AMPS by precipitation with an ambiphilic quaternary amine[2,6] results in excellent chromatographic (or electrophoretic) separations of the different compounds. Even without a desalting process, TLC still provides a rapid means of detecting an elevation of urinary AMPS. Furthermore, by simple variations of technique it is possible to quantify the total amount of AMPS in a urine and make a preliminary identification of what substances are present.

METHODS

The diurnal variation of AMPS excretion necessitates the use of an aliquot from a 24-h collection. Using a Nichrome wire loop[7] calibrated to deliver about 800 nl (ref. 8), approximately 1.6 μl (two loops) of raw urine is applied incrementally as a circular origin spot 10 mm from the bottom edge of a 10 cm high $\times$ 5–10 cm wide sheet of an Eastman Kodak No. 13255 cellulose Chromagram. Samples and one and two loop volumes of dermatan sulfate (35 μg/ml) are applied 20 mm apart and from each edge. The solvent system of Humbel *et al.*[6] is used, *i.e.* 0.4 *M* ammonium formate–absolute methanol (45:55), containing 1.1 m*M* ethylenediaminetetracetic acid. After the solvent has risen to the top edge, the plate is removed and dried thoroughly in a stream of cool air. The AMPS are visualized by immersing the chromatograms in 0.5% toluidine blue in 5% acetic acid for 5 min. The excess dye is drained off and the

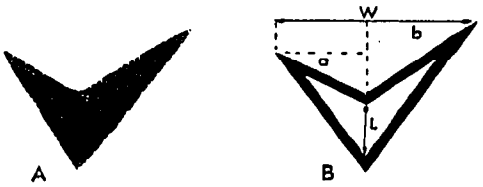

Fig. 1. (A) Drawing of typical AMPS spot illustrating the chevron or concave quadrilateral shape. (B) Calculation of the area of the spot in Fig. 1A: L is the base of both triangles, W is the sum of the two altitudes a and b and the sum of the area of the two triangles is $\frac{1}{2} L \cdot W$.

background is cleared by immersing the plate in absolute methanol for 10 min. The chromatogram is air dried for evaluation.

In this procedure the AMPS are not resolved and appear as a single chevron-shaped spot (Fig. 1A) from which the total concentration of urinary AMPS can be determined by means of the product of the spot area and maximum absorbance[9,10]. The maximum absorbance is measured as described previously[10], but determination of the area requires a modification of the earlier procedure. The chevron-shaped spots can be regarded as two obtuse triangles with one side (the base) in common (Fig. 1B). By simple geometric analysis, the area of the figure can be shown to consist of half the product of the base and the sum of the two altitudes (Fig. 1B). In some cases the spots more closely resemble crescents (Fig. 2), in which case the area can be obtained from

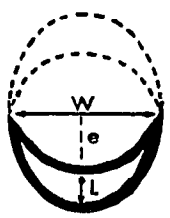

Fig. 2. Area of a crescent shape spot. The area of the semi-circle of diameter W is $(\pi/8)W^2$. The area of the half ellipse is $(\pi/4)W(L+e)$, where $e = W/2$ or $(\pi/8)\ (W^2+2LW)$. The area of the crescent is the difference of these two figures or $(\pi/4)\ (L \cdot W)$.

the difference between that of the semi-circle and the half-ellipse. This is a function of the same two linear measurements as in the concave quadrilateral (*cf.* Figs. 1B and 2). For quantification dermatan sulfate standards, 50–300 μg/ml in normal urine are applied in the same volumes as the unknown. The latter is spotted in triplicate.

If the urine samples and standards are applied to the chromatogram as thin lines about 25 mm long and the chromatography is conducted as described above, the AMPS appear as poorly resolved bands which aid in identification but are too poorly separated for quantitative measurement. The relative migration of the individual AMPS is the same as reported previously[6].

RESULTS

The technique has been used to detect patients with Hurler's, Morquio's and San Fillipo's syndromes. The initial TLC screening followed by chromatography of band-shaped spots has provided a rapid means of identifying those patients requiring

more sophisticated chemical analyses[11] or skin biopsies and studies of the cultured fibroblasts[12]. Spotting, chromatography and color development require about 40 min per plate.

The quantification technique has been useful for following the urinary excre-

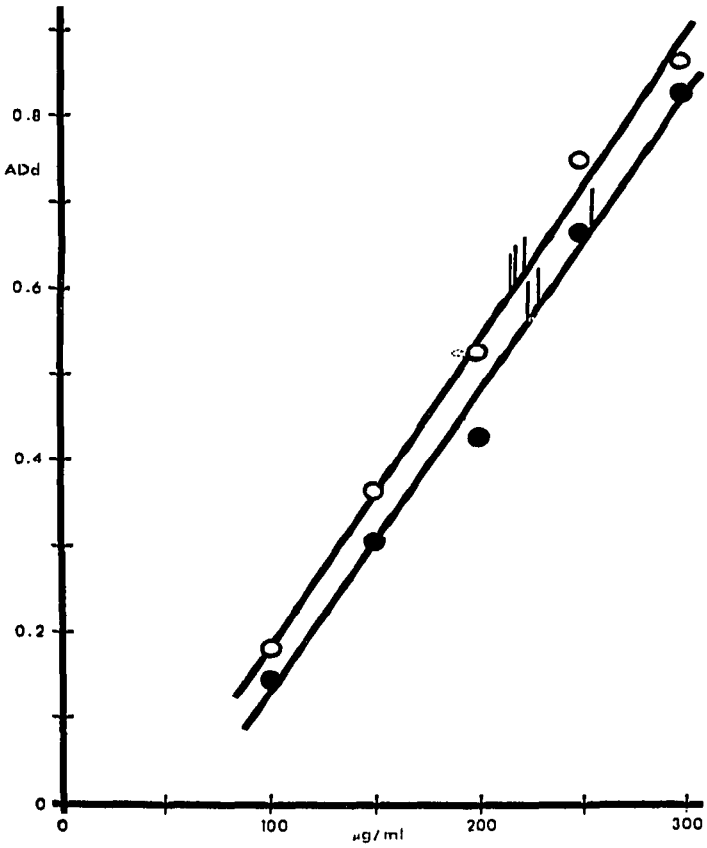

Fig. 3. Standard curves and superimposed observations of duplicate analyses of urinary AMPS from a patient with Hurler's syndrome. ADd is the triple product of the spot absorbance and two dimentions (ref. 10)

tion of AMPS in patients receiving various treatments (Fig. 3). The method demonstrated that "irregular" spots can be quantified provided they are geometrically similar.

DISCUSSION

In both the chevron- and crescent-shaped spots, the area is a function of ($L \times W$) but the proportionality constants are quite different. Therefore, it is essential that chromatographic spots of standards and unknowns are geometrically similar. Ordinarily, on a small chromatogram they are quite uniform. Concave spots have "horns" of variable length; measurement of the spot dimensions is difficult and somewhat arbitrary but for any given investigator it can be quite reproducible and accurate. It

is doubtful if chromatograms of this sort could be quantified as accurately by any other method. Past experience has shown that area or maximum absorbance alone do not have the precision of the product of the two determinations[10,13].

The salt content of urine causes a retardation and distortion of the AMPS spots which is ordinarily overcome by an initial purification of the AMPS. An alternate approach is to prepare the reference standards in a normal urine so that the chromatographic spots are similarly affected. Unless the unknown and standard spots have a similar R_F value and geometric shape, accurate quantification is not possible.

As a screening technique this method is simple, rapid and reliable. It cannot be used to render a specific diagnosis but that is not its purpose. It can identify those individuals who do require one of the costly, time-consuming procedures required for differential diagnosis while weeding out the patients who do not.

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