CHROM. 5524

Separation of sugars from untreated urine

Desalting of the sample is an essential preliminary step in the paper chromatography of urinary sugars. High voltage electrophoresis, which aims at eliminating this step, has not been very successful in sugar chromatography[1]. In the method described in this paper, the common urinary sugars can be separated as compact spots from 100 μl of untreated urine spotted on Whatman 3MM filter paper. The method is based on the principle (discussed previously[2] in connection with a method for paper chromatographic separation of amino acids from untreated urine), that contaminants cannot adversely influence the discrete separation of compounds, if the latter have speeds of migration that are different from those of the contaminants in a chromatographic run.

In 90% pyridine, sugars migrate much faster (all common sugars have R_F values of 0.65) than the contaminants (urea, $R_F = 0.45$; sodium chloride, $R_F = 0.15$; alanine, $R_F = 0.18$) and, therefore, sugar spots are not much distorted by the presence of these contaminants. Thus, from a urine spot (100 μl), in a short ascending run in 90% pyridine (the desalting run) it is possible to obtain a compact composite spot of sugars out of influence of the common urinary contaminants for any subsequent run at right angles to this desalting run.

Experimental

One-dimensional separation. Urine corresponding to 0.1 mg of creatinine is spotted, in a conventional manner, in one corner of a 46 × 19 cm sheet of Whatman 3MM filter paper. The spot is 1 cm from the long edge of the paper and 5 cm from its broad edge.

The desalting step. The paper is subjected to two consecutive ascending runs (20 min, 90 min) in 90% aqueous pyridine along its breadth. The various urinary sugars form a composite spot near the solvent front, well separated from the large urea spot. The important urinary amino acids and salts remain far behind the urea spot. The paper is then cut along its length, midway between the solvent front line and the position of the urine spot. The segment bearing the urine spot is discarded and the other segment is run along its length for the separation of the sugar spots.

Run for separating the spots. Any conventional sugar solvent can be used in a descending run. In this laboratory, the solvent mixture isopropyl alcohol–*n*-butyl alcohol–*tert.*-butyl alcohol–water (4:2:2:2) (solvent A) is used, first in a 2-h ascending run and then in a 16-h descending run. The 2-h run can be omitted without much disadvantage to the separations. After drying in air, the chromatograms are stained with *p*-aminobenzoic acid reagent[3].

Two-dimensional separation. In this case, the urine is spotted on a 46 × 28 cm sheet of Whatman 3MM filter paper, the spot being 1 cm from the broad edge and 2 cm from the long edge of the paper. The desalting runs are conducted along the length of the sheet. After the runs, part of the paper is cut away as described above for the desalting step in the one-dimensional procedure. The paper bearing the composite sugar spot is subjected to a two-dimensional separation described below.

Runs for separation of the spots. The first run (ascending, 8 h) is at right angles

to the direction of the desalting runs. The solvent mixture phenol–water–ammonia (160:40:1, w/v/v) (solvent B) is used for this run[4]. After the run, the papers are dried under a fan. A cut is made 3 cm below and parallel to the solvent front line and the segment bearing the dark solvent front is discarded. The final descending run (16 h) in solvent A is in the direction of the initial desalting runs. The papers are dried and stained as described for the one-dimensional chromatograms.

Results and discussion

Pyridine extraction procedures for desalting sugar samples have been criticised[4] since they result in chemical transformations in some sugars. This is especially true for methods in which hot pyridine is used. Pyridine-containing solvents, however, are widely employed in the paper chromatography of sugars. In the present method, only a pyridine-containing solvent has been used for the desalting step.

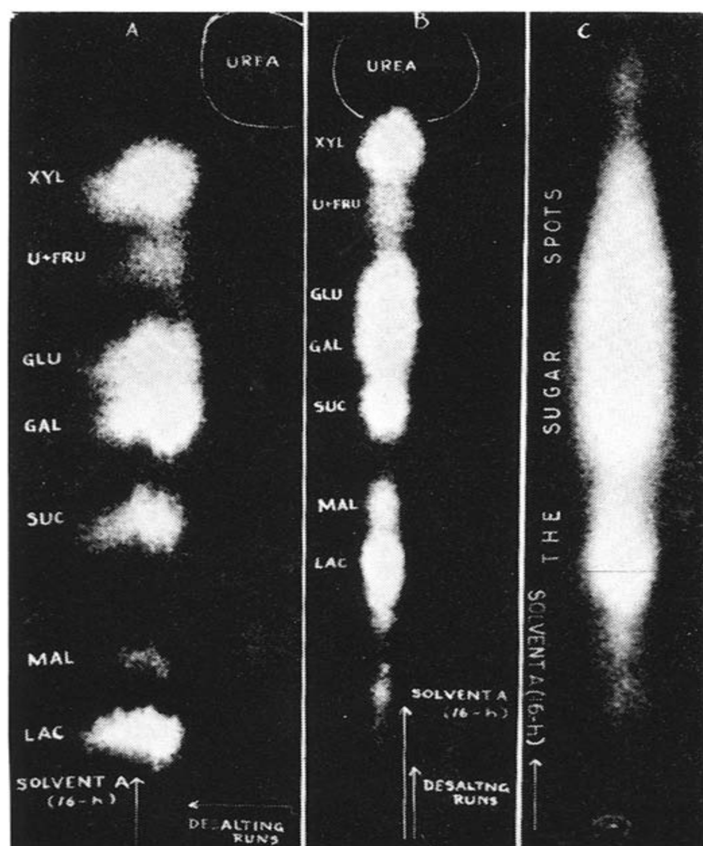

Fig. 1. Segments of the one-dimensional chromatograms for urinary sugars on Whatman No. 3MM filter paper. For chromatogram A on a 46 × 19 cm sheet, 100 μl of urine and the standard sugar solution were spotted in one corner 1 cm from the long edge and 5 cm from the broad edge. For chromatograms B and C on 46 × 10 cm sheets, 100 μl of urine and the standard solution were spotted in a conventional way near the smaller edge. The sequence of runs for chromatogram A was along the breadth (the desalting runs) and along the length (solvent A). In this sequence, urea does not come into contact with the sugar spots in the run in solvent A. The sequence of runs for chromatogram B was along the length (the desalting runs) and again along the length (solvent A). In this sequence, urea crosses over the various sugar spots in the run in solvent A. In the case of chromatogram C, the desalting runs have been omitted and the paper is only run along its length in solvent A. Desalting runs: 90% pyridine, ascending, 20 min, 90 min. Solvent A: isopropyl alcohol–*n*-butyl alcohol–*tert.*-butyl alcohol–water (4.2:2:2), descending, 16 h. U indicates an unknown sugar spot, staining red with *p*-aminobenzoic acid reagent.

Recoveries of all common urinary sugars (fructose, glucose, galactose, lactose, maltose, sucrose and xylose) are greater than 95% in the desalting step of the procedure described. In sugar chromatography, the conventional desalting procedures for urinary samples do not eliminate urea or amino acids. The present method has the advantage of removing the interference due to these compounds.

In the desalting run in 90% pyridine, the R_F value of urea is less than that of the composite sugar spot while the R_F value of urea is greater than those of the common sugars in solvents A and B. Therefore, in the recommended one-dimensional method, where the run for separating the sugar spots in solvent A is at right angles to the direction of the desalting runs, there is no contact between the urea and the sugar spots in solvent A. However, if the run in solvent A is conducted in the direction of the desalting runs, it is apparent that a large urea spot will cross over the various sugar spots during the run. According to the principle quoted in the introductory remarks, this should result in elongation of the sugar spots. Some elongation of the

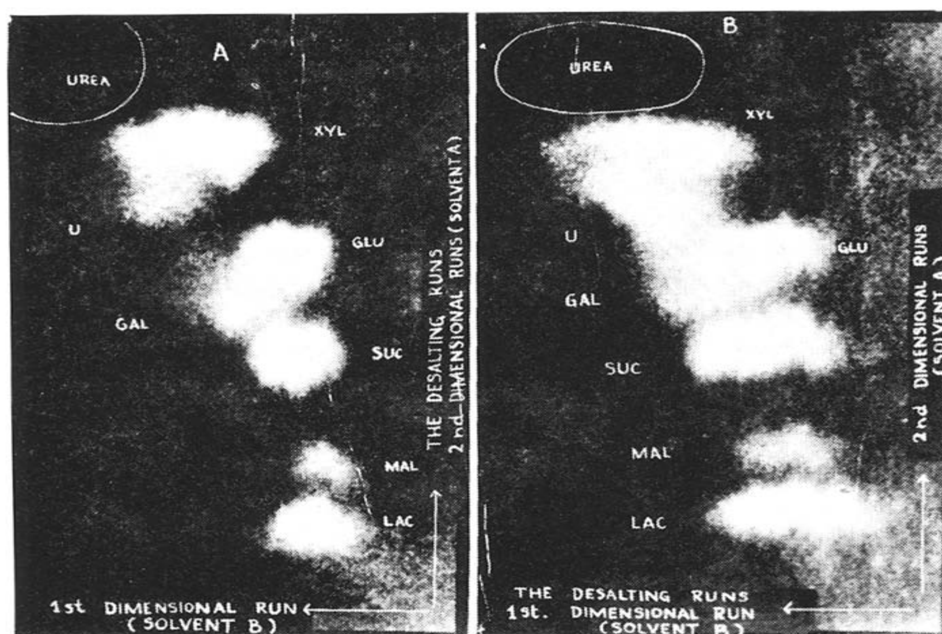

Fig. 2. Segments of the two two-dimensional sugar chromatograms on Whatman No. 3MM filter paper demonstrating the effect of contaminant (urea) crossing over the sugar spots during a run. For chromatogram A, on a 46 × 28 cm sheet, 100 µl of urine and the standard sugar solution (containing lactose, maltose, sucrose, galactose, glucose and xylose) were spotted in one corner 1 cm from the broad edge and 2 cm from the long edge. For chromatogram B, on a 46 × 42 cm sheet, 100 µl of urine and the standard sugar solution (containing the sugars present in the standard solution for chromatogram A and also fructose) were spotted in one corner 1 cm from the 46-cm edge and 4 cm from the 42-cm edge. For chromatogram A, the sequence of the runs was along the length (the desalting runs), along the breadth (solvent B) and along the length again (solvent A). In this sequence, urea does not come into contact with the sugar spots in the run in solvent B. For chromatogram B, the sequence of runs was along the breadth (the desalting runs), along the breadth again (solvent B) and along the length (solvent A). In this sequence, urea crosses over the various sugar spots in the run in solvent B. Desalting runs: 90% pyridine, ascending, 20 min, 90 min. Solvent A: isopropyl alcohol–*n*-butyl alcohol–*tert*.-butyl alcohol–water (4:2:2:2), descending, 16 h. Solvent B: phenol–water–ammonia (160:40:1, w/v/v), ascending, 8 h. U indicates an unknown spot staining red with *p*-aminobenzoic acid reagent. This spot overlaps the position of the fructose spot. In chromatogram B, although not indicated, the spot U actually indicates the above unknown spot + fructose.

spots does occur under these circumstances when urea crosses over the sugar spots in solvent A (Fig. 1). However, the effect is more striking if, in the two-dimensional method, the run in solvent B (the phenol solvent) is conducted in the direction of the desalting runs thus making urea cross over the sugar spots in the phenol solvent (Fig. 2). This appears to indicate that in a situation where a contaminant migrates close to the actual compounds (or passes through them), the distorting effect will depend on the nature of the solvent. One determining factor might be how compactly the contaminant (in the amounts used) moves in a particular solvent. Thus, the greater elongation of the sugar spots observed in the two-dimensional method can be attributed to the fact that urea migrates less compactly in the phenol solvent B compared with solvent A. Urea spots (0.3 cm diameter, 2 mg amount) on Whatman No. 3MM filter paper, when subjected to 6-h descending runs in the two solvents, had final sizes of 4.0 × 2.1 cm and 3.2 × 2.4 cm in solvents B and A, respectively, showing that urea migrates more compactly in solvent A than in the phenol solvent.

It follows that in a solvent in which both the actual compounds and the contaminant move in a compact fashion, there is less likelihood of their mutual interference even if they migrate close together. Most of the solvents used in the chromatography of sugars and amino acids are excellent solvents for urea as well (but not for sodium chloride and other salts). This is probably the reason why, in the chromatography of urine samples (generally containing more urea than salts), greater stress is laid on the elimination of salts than of urea.

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Department of Physiology, Medical College,
Rohtak, Haryana (India)

A. S. SAINI

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