Journal of Chromatography, 95 (1974) 51–58

CHROM. 7436

A TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHIC METHOD FOR SCREENING CARBOHYDRATE ANOMALIES

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(Received March 15th, 1974)

SUMMARY

A two-dimensional thin-layer chromatographic method is described for the identification of clinically important carbohydrates in urine. On the same plate, glucose, galactose and levulose are also separated in human plasma by a one-dimensional technique.

The application of untreated urine and plasma, the possibility of identifying with accuracy the carbohydrates present in the samples and the rapidity of the procedure make this method particularly suitable for the screening of carbohydrate anomalies.

INTRODUCTION

In order to screen the errors in carbohydrate metabolism, it is necessary to have a simple and rapid method that permits the detection of abnormal concentrations of sugars in urine and plasma. For this purpose, it is obvious that chromatography offers the best method of separating and analyzing several sugars at the same time.

In recent years, many workers[1–17] have used thin-layer (TLC), gas–liquid (GLC) and ion-exchange chromatography for carbohydrate analysis; and various methods have been developed for the separation of mixtures of sugars in pure solutions. However, the application of these methods to the study of urinary or plasma carbohydrates requires long and laborious processes of purification, desalination and concentration of the samples before they can be subjected to chromatographic analysis[18–21].

On the other hand, in order to simplify screening and to reduce its time of execution, the samples need not be pre-treated. The work of Jolley and co-workers[22–25] cannot be applied because their chromatographic system is time consuming and they used procedures that are too complex for use in routine analyses.

Other workers[26–28] have developed TLC methods that permit the analysis of untreated urine, but the separation of the sugars has not been very satisfactory.

The method of Krafficzick *et al.*[29] also has considerable limitations: (a) the chromatographic procedure needs too much time as a double development in each direction is necessary; (b) the use of internal standards, besides falsifying the natural

constitution of the sample, is useless in identifying the spots in the absence of R_F values or other criteria; and (e) the colour difference alone is an unreliable method for the identification of the spots.

In recent work we have used the "dual-layer technique" studied by other workers in the resolution of complex mixtures of substances[30-36] and we developed a chromatographic method for the separation of a complex mixture of carbohydrates[8]. Although in many analyses performed in this laboratory we observed that this method did not require pre-treated biological samples, it is inapplicable to clinical screening tests because of the length of time required for the chromatographic process and of the complexity of the procedures.

In this paper we describe a method that has given results comparable with those from the "dual-layer technique" and that is capable of being applied extensively in routine analysis. In this method the urine is not pre-treated, the separation is achieved in a very short time and the identification of the sugars is carried out without the use of internal standards.

EXPERIMENTAL AND RESULTS

Preparation of the plates

Silica gel G-60 (Merck, Darmstadt, G.F.R.) and Syloid 63 (W. R. Grace, Baltimore, Md., U.S.A.), are accurately mixed in a 2:1 ratio, then 30 g of this mixture are slurried with 60 ml of an aqueous solution of 0.032 *M* sodium tetraborate and 0.05 *M* sodium tungstate (3:1) and spread over six glass plates (20 × 20 cm) in the usual way so as to obtain 300-μm thick layers. The coated plates are dried in an oven at 90° for 30 min.

Solvent systems

For the first run we used the neutral solvent system ethyl acetate–isopropanol–water (2:2:1) used in our previous work, and for the second run the acidic solvent system ethyl acetate–methanol–acetic acid–water (60:15:15:10).

Chromatographic procedures

The gel surface is divided into four sectors (as indicated in Fig. 2) by means of two orthogonal lines. The largest sector (12 × 12 cm) is used for the two-dimensional chromatography. In this sector, 10–15 μl of untreated urine are deposited at a distance of 1 cm from each external side by means of a 2-μl pipette, dried with a stream of warm air between applications and overspotted with 1 μl of 0.1 *N* silver nitrate solution. Then 10 μl of plasma are applied as a 2-cm long line in sector II (Fig. 2). The plates are developed once in the neutral solvent (2 h) and then dried in an oven at 70° for 4–5 min. The second development, at right angles to the first, is carried out in the acidic solvent (50 min). In each instance unsatured chambers are used.

Detection and identification

After the final development, sugars are detected by spraying with a 0.2% solution of naphthalene-1,3-diol in ethanol–concentrated sulphuric acid (95:5) and heating the plates at 105° for 5 min.

Typical separations of standard carbohydrates mixed with normal human urine

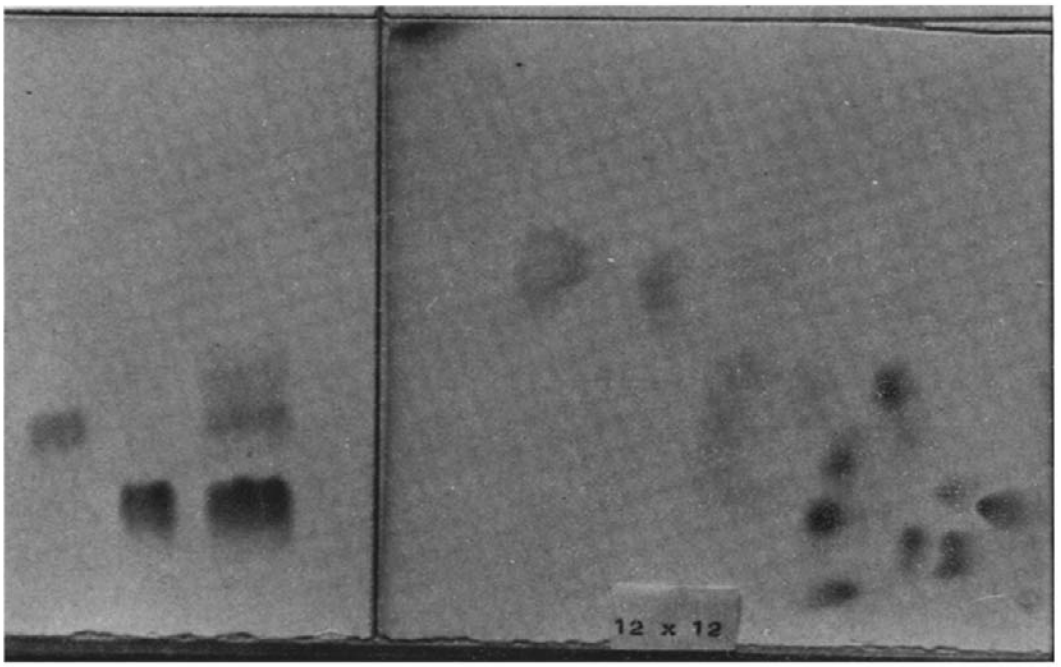

Fig. 1. Two-dimensional separation of carbohydrates in urine and, on the left-hand side, the one-dimensional chromatography of galactose and fructose-loaded human plasma.

are shown in Fig. 1 and, on the left-hand side, the one-dimensional chromatogram of plasma carbohydrates of clinical interest is shown.

Three criteria are used for the identification of the spots: (a) the R_F values of each spot in the two dimensions; (b) the characteristic colours developed by each sugar (ketoses and ketose-containing oligosaccharides generally give red spots and hexoses yield blue colours (Table I); and (c) the subdivision of the chromatogram into four sectors (A, B, C and D) by means of two straight lines, one passing through the origin and the centre of the urea spot and the other perpendicular to the lower edge of the plate and tangential to the sucrose spot (Fig. 2). Oligosaccharides migrate in sectors A and B. Sector C contains the spots of galactose, glucose, sedoheptulose, fucose and 2-deoxyribose, while xylose, ribose, fructose, xylulose and allulose occur in sector D.

DISCUSSION

This chromatographic method should be considered as an alternative to the "dual-layer technique" that Berger and co-workers[30-34] and other workers[8,35,36] devised in order to obtain better separations of complex mixtures of substances using two different chromatographic processes on the same plates. In fact, as is shown in Fig. 3, in the first run, in which the neutral solvent is used, the sodium tetraborate-impregnated silica gel exhibits a different chromatographic behaviour from that displayed in the second run with the acidic solvent.

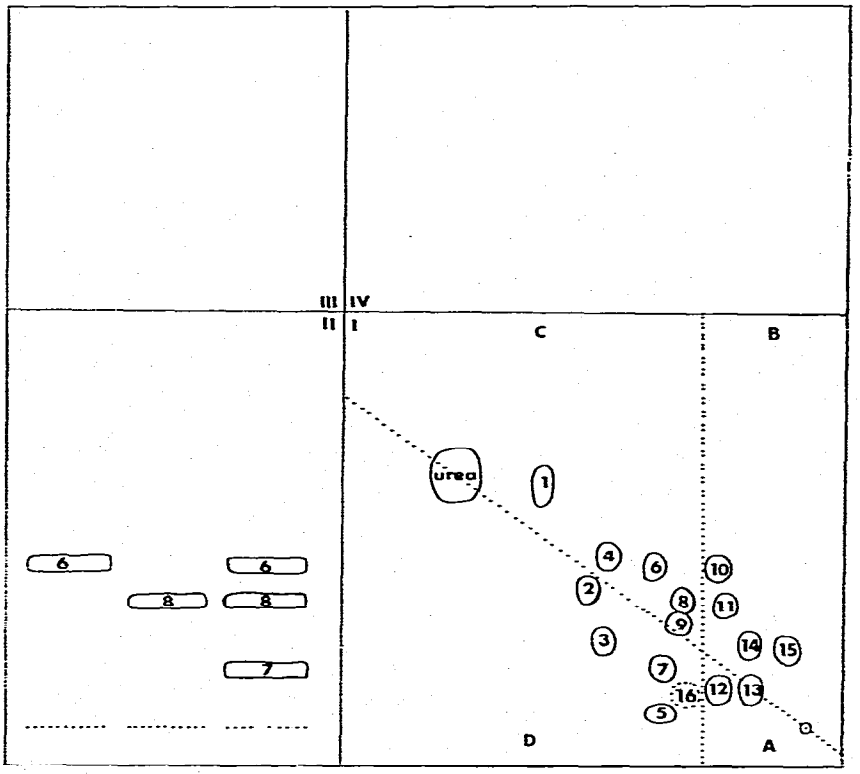

Fig. 2. Thin-layer chromatogram of urea and sugars that commonly occur in human urine (two-dimensional) and in plasma (one-dimensional) separated on sodium tetraborate–sodium tungstate-impregnated silica gel plates. Solvent systems: ethyl acetate–isopropanol–water (2:2:1) and ethyl acetate–methanol–acetic acid–water (60:15:15:10). Spots: 1 = 2-deoxyribose; 2 = xylose; 3 = ribose; 4 = fucose; 5 = xylulose; 6 = glucose; 7 = fructose; 8 = galactose; 9 = sedoheptulose; 10 = sucrose; 11 = maltose; 12 = palatinose; 13 = lactulose; 14 = lactose; 15 = raffinose; 16 = allulose.

In particular, in the first development the chromatographic separation appears to be due primarily to sorption–desorption processes through the complexing of the sugar molecules with the tetraborate ions, which are held on the silica matrix, and their subsequent decomplexing. In this system, therefore, the sugars are mainly resolved according to their capacity to form borate complexes and only to a small extent according to their partition coefficients. On the contrary, in the second run, the impregnating agent is completely deactivated by the acidic solvent and the carbohydrate separation is achieved through a normal partition process. As confirmation of the above, the chromatographic pattern of the sugars separated by the second solvent appears to be the same whether impregnated plates are used or not (Fig. 4).

However, the neutral solvent, when used on unimpregnated plates, tends to separate the sugars according to their solubilities and it is completely unsuitable for the separation of even very simple mixtures (Fig. 5).

The interference of several urinary organic components and inorganic salts in the separation of carbohydrates is considerable in paper and thin-layer chromatogra-

TABLE I

$R_F \times 100$ VALUES, COLOUR REACTIONS AND SENSITIVITY LIMITS OF THE CARBOHYDRATES STUDIED AND OF UREA

Compound	$R_F \times 100$		*Colour*	*Sensitivity limit* (%)*
	First Solvent	*Second Solvent*		
Urea	59	76	Orange	—
2-Deoxyribose	58	56	Pea green	5
Xylose	33	46	Brown	10
Ribose	20	44	Light blue	10
Fucose	41	42	Red	10
Xylulose	4	30	Orange	2
Glucose	38	33	Violet	20
Fructose	15	30	Purple-red	2
Galactose	29	27	Blue	10
Sedoheptulose	23	28	Purple-red	2
Sucrose	38	20	Red	4
Maltose	29	18	Blue	20
Palatinose	9	18	Red	4
Lactulose	9	12	Red	4
Lactose	20	12	Blue	10
Raffinose	17	4	Purple-red	5
Allulose	7	25	Red	—

* Minimum amount of sugar (mg) detected in 100 ml of urine sample.

phy, in which purification is an essential stage of the analysis. The application of our chromatographic system obviates the interferences due to inorganic salts, urea, pigments, creatinine, uric acid, other organic acids and amino acids, mainly because the impregnation of silica gel with salts capable of complexing the sugars selectively results in their migration becoming independent of that of the other non-saccharidic substances.

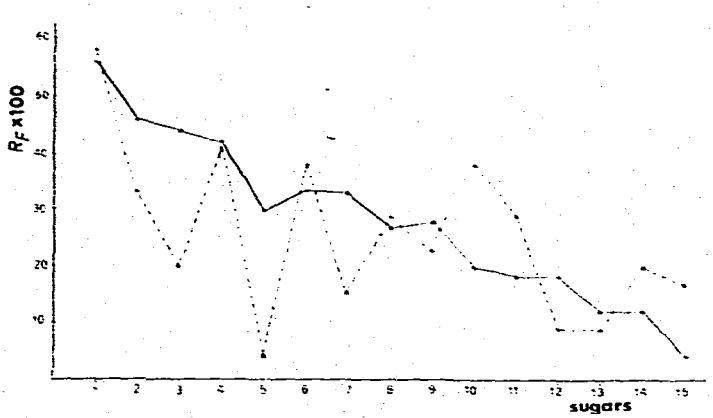

Fig. 3. Plots of $R_F \times 100$ values of carbohydrates separated on sodium tetraborate–sodium tungstate-impregnated silica gel plates. The sugars on the abscissa are enumerated according to the increasing number of hydroxyl groups. Broken line, neutral solvent; solid line, acidic solvent.

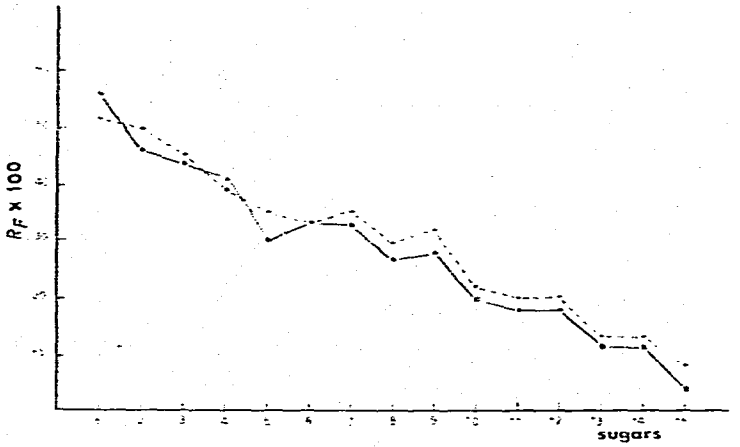

Fig. 4. Plots of $R_F \times 100$ values of carbohydrates on unimpregnated and sodium tetraborate-sodium tungstate-impregnated silica gel plates, separated by the acidic solvent. Broken line, water; solid line, sodium tungstate.

We believe, in fact, that when the chromatographic plate is impregnated, a discontinuous but homogeneous distribution of different sites is formed on the gel surface and these sites hold the impregnants in different concentrations. Tetraborate-rich sites should be more accessible to the saccharide molecules, for which they have a particular affinity.

Furthermore, as the over-spotting of the pre-spotted urine samples with silver nitrate solution retains a large amount of chloride ions (which are the major interferents) as insoluble silver chloride on the origin, the adverse influence of these ions on the migration of sugars is limited. Moreover, under the conditions used, the silver nitrate does not seem to exert its oxidative properties on the carbohydrates. It is there-

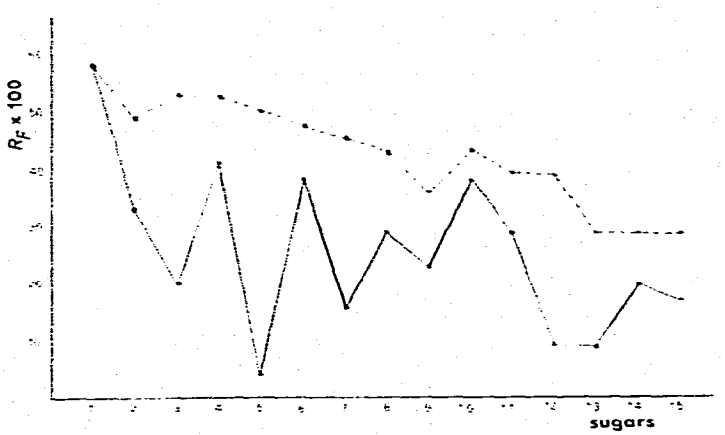

Fig. 5. Plots of $R_F \times 100$ values of carbohydrates on unimpregnated and sodium tetraborate-sodium tungstate-impregnated silica gel plates, separated by the neutral solvent. Broken line, water; solid line, sodium tungstate.

fore possible to spot on the plate 10–15 μl of untreated urine without observing any adverse effects on the carbohydrate chromatography.

With the amount of urine sample applied on the plate, urea becomes easily visible besides sucrose, which is always present in normal human urine as demonstrated by other workers[25-37] and by our own observations (unpublished work). These two spots are particularly useful for the identification of the sugars on the chromatogram, so that we can exclude the use of the internal standards.

Fructose and allulose are the other carbohydrates that are frequently found in normal human urine by our method, together with two or three blue spots, which are probably steroids. It should be noted that fructose shows a marked sensitivity to the reagent. Thus a normal diet fructosuria could easily be mistaken for a metabolic anomaly of this sugar. In this case, one-dimensional chromatography of plasma on the same plate on which urine analysis is performed would dispel any doubt.

In conclusion, the chromatographic method described offers a means of recognizing the mellituries of enzymopathological origin in children, through the identification of the affected carbohydrate.

REFERENCES

1 A. Affonso, *J. Chromatogr.*, 27 (1967) 324.
2 K. Linek, L. Kuniak and B. Alince, *Chem. Zvesti*, 21 (1967) 99.
3 J. P. Marais, *J. Chromatogr.*, 27 (1967) 321.
4 S. M. Petrovic and V. D. Canić, *Microchim. Acta*, (1969) 599.
5 M. Tomada, *Yakugaku Zasshi*, 87 (1967) 207; *C.A.*, 67 (1967) 11685m.
6 M. Lato, T. Mezzetti, B. Brunelli and G. Ciuffini, *J. Chromatogr.*, 39 (1969) 467.
7 T. Mezzetti, M. Lato, S. Rufini and G. Ciuffini, *J. Chromatogr.*, 63 (1971) 329.
8 T. Mezzetti, M. Ghebregzabher, S. Rufini, G. Ciuffini and M. Lato, *J. Chromatogr.*, 74 (1972) 273.
9 Th. Kartnig and O. Wegschaider, *J. Chromatogr.*, 61 (1971) 375.
10 P. E. Reid, B. Donaldson, D. W. Secret and B. Bradford, *J. Chromatogr.*, 47 (1970) 199.
11 D. Farshtchi and C. W. Moss, *J. Chromatogr.*, 42 (1969) 108.
12 J. P. Zanetta, W. C. Breckenridge and G. Vincendon, *J. Chromatogr.*, 69 (1972) 291.
13 R. M. Saunders, *Carbohyd. Res.*, 7 (1968) 76.
14 E. F. Walborg, Jr., D. B. Ray and L. E. Ohrenberg, *Anal. Biochem.*, 29 (1969) 433.
15 H. G. Walker, Jr., and R. M. Saunders, *Cerial Sci. Today*, 15 (1970) 140.
16 J. S. Hobbs and J. G. Lawrence, *J. Chromatogr.*, 72 (1972) 311.
17 E. Martinsson and O. Samuelson, *J. Chromatogr.*, 50 (1970) 429.
18 V. Vitek and K. Vitek, *J. Chromatogr.*, 60 (1971) 381.
19 M. Lato, B. Brunelli, G. Ciuffini and T. Mezzetti, *J. Chromatogr.*, 36 (1968) 191.
20 M. Paget and P. Coustenoble, *Ann. Biol. Clin. (Paris)*, 25 (1967) 1239; *C.A.*, 68 (1968) 102430m.
21 V. K. Gorodetskii, *Sovrem. Metody. Biokhim.*, (1968) 350; *C.A.*, 72 (1970) 75458a.
22 R. L. Jolley and M. L. Freeman, *Clin. Chem.*, 14 (1968) 538.
23 R. L. Jolley, K. S. Warren, C. D. Scott, J. L. Jainchill and M. L. Freeman, *Amer. J. Clin. Pathol.*, 53 (1969) 793.
24 C. D. Scott, R. L. Jolley and W. F. Johnson, *Amer. J. Clin. Pathol.*, 53 (1970) 701.
25 R. L. Jolley and C. D. Scott, *Clin. Chem.*, 16 (1970) 687.
26 A. S. Saini, *J. Chromatogr.*, 61 (1971) 378.
27 D. S. Young and A. J. Jackson, *Clin. Chem.*, 16 (1970) 954.
28 C. Metras, *Can. J. Med. Technol.*, 29 (1967) 121.
29 F. Kraffczyk, R. Helger and J. Bremer, *Clin. Chim. Acta*, 42 (1972) 303.
30 J. A. Berger, G. Meyniel, P. Blanuqet and J. Petit, *C.R. Acad. Sci.*, 257 (1963) 1534.
31 J. A. Berger, G. Meyniel, G. Petit and P. Blanquet, *Bull. Soc. Chim. Fr.*, (1964) 3179.

32 J. A. Berger, G. Meyniel and J. Petit, *Bull. Soc. Chim. Fr.*, (1963) 2662.
33 J. A. Berger, G. Meyniel and J. Petit, *C.R. Acad. Sci.*, 259 (1964) 2231.
34 J. A. Berger, G. Meyniel and J. Petit, *J. Chromatogr.*, 29 (1967) 190.
35 J. Petit, J. A. Berger, G. Gaillard and G. Meyniel, *J. Chromatogr.*, 39 (1969) 167.
36 J. G. Kirchner, *J. Chromatogr.*, 63 (1971) 9.
37 V. K. Gorodetskii, V. I. Mikhailoff and E. L. Rosenfeld, *Clin. Chim. Acta*, 34 (1971) 67.