Journal of Chromatography, 117 (1976) 211–215

CHROM. 8770

Note

Reversed-phase high-pressure liquid chromatography of normal rat urinary carbohydrates

RICHARD B. MEAGHER S. J. and ARTHUR FURST

Institute of Chemical Biology, University of San Francisco, San Francisco, Calif. 94117 (U.S.A.)

(Received September 29th, 1975)

In our investigation of differences of rat-excreted urinary carbohydrate profiles between normals and those with pathological conditions, a procedure was sought that required less time than the gas–liquid chromatographic (GLC) techniques[1–5] and that involved simpler apparatus than that used in the automatic anion-exchange high-resolution column chromatography[6–8]. High-pressure liquid chromatography (HPLC) has been reported to be useful in separating a known mixture of sugars[9] and in separating saccharides in beet syrups[10]. These latter procedures were modified to process rat urine in order to obtain the profile of sugars excreted.

EXPERIMENTAL

Apparatus and materials

A Waters Assoc. Liquid Chromatograph ALC CPC (201), equipped with a Model-600 Solvent Delivery System and a differential refractometer detector (1×10^8 refractive index units), was used; the attenuation was 128–1/4. An E&K Strip Chart Recorder Model 255, with chart speeds 0.5, 1, 2, 8, 16 in./min or 0.5, 1, 2, 8, 16 in./h, was attached; the usual speed was 16 in./h. The column was μBondapak-carbohydrate (Waters Assoc.), 30 cm × 4 mm I.D. stainless steel. The eluent was water–acetonitrile (15:85) and water–acetonitrile (25:75). The sample size was usually 25 μl, which was injected with a syringe (Precision Sampling, Baton Rouge, La., U.S.A.) via a Model U6K Universal Injector (Waters Assoc.), which can handle samples from 1 μl to 2 ml without modification. Other apparatuses used were: American Optical Total-Solid (TS) meter, Model 10400; Bio-Rad Bio-Fiber Minibeaker 50; Bio-Rad Bio-Fiber Stir Module, Model 620; Millipore all-glass filter apparatus; and Millipore pre-filters and aqueous filters. The chemicals used were: acetonitrile, distilled in glass (Burdick and Jackson Labs., Muskegon, Mich., U.S.A., for most work; Mallinkrodt, St. Louis, Mo., U.S.A., for some)*; glycerol (J. T. Baker, Phillipsburg, N.J., U.S.A.) and myoinositol (N.B.C., Cleveland, Ohio, U.S.A.). These latter two were used as the polyol standards. The carbohydrate standards were purchased from Sigma (St. Louis, Mo., U.S.A.) with the exception of the following: xylose and sucrose from J. T. Baker,

* The elution times were somewhat dependent upon the source of the acetonitrile.

L-arabinose from Eastman-Kodak (Rochester, N.Y., U.S.A.) and fructose from Matheson, Coleman and Bell (East Rutherford, N.J., U.S.A.).

Preparation of rat urine samples

In a 24-h period, a normal, mature Fischer-344 male rat excreted about 7 ml of urine which was collected and centrifuged to sediment out any extraneous particles that might have come from the metabolic cage. The urine was tested with a Combistix that indicated the presence of at least 100 mg of protein per 100 ml, the absence of glucose and a pH of 6. As rat urine is hypergravic, no reading could be obtained in the urine total-solid part of the scale of the TS meter. However, a reading was obtained on the plasma–serum scale, and an estimation of total solids concentration in urine was made from that reading. The 24-h rat urine was diluted to 10 ml, and 8 ml of the diluted urine was used to fill a Bio-Fiber 50 Minibeaker. The urine was dialyzed with 50 ml of deionized water. The dialysate was concentrated to 10 ml in a vacuum rotary evaporator and passed through a 0.2-μm pore-sized Metricel filter to remove all particulate matter.

A drop of the dialysate (the aqueous solution of the substances that passed through the Bio-Fibers) was examined on the TS meter. The dialysate had a refractive index of 1.3390, which indicated a non-protein total solids concentration of 5.85 g per 100 ml. (When not in use, the dialysate must be kept frozen to prevent formation of particles.)

Procedure

The operations were carried out at ambient temperature. A water–acetonitrile mixture was always the eluent. The solvent ratio, flow-rate and attenuation were always selected according to the information sought in the chromatogram. Table I indicates what selections are to be made for obtaining information.

TABLE I

EXPERIMENTAL CONDITIONS FOR OBTAINING DIFFERENT SUBSTANCES

Purpose of chromatogram	*Water–acetonitrile ratio*	*Flow-rate (ml/min)*	*Attenuation*
To quantitate urea and glycerol in dialysate	15:85	2	16×
To obtain one chromatogram profile for all carbohydrates and to quantitate monosaccharides	15:85	2	2×
To study and prepare standards for only the disaccharides, myoinositol and oligosaccharides in dialysate	25:75	2	2×
To produce chromatogram at very low concentrations and to avoid noise			1/2× or 1/4×

REPRODUCIBILITY

With care, reproducible results can be obtained. Maintaining the solvent ratio is important, because any change in ratio involves changes in elution times. Linden and Lawhead[10] have pointed out that, after four months of continuous use, differences

in column performance were noted. This may explain why some columns show elution times different from others. For our present purposes, we did not find it necessary to identify all the peaks appearing after raffinose in the chromatograms.

This procedure is very valuable in our work in obtaining the rat urinary carbohydrate profiles; the number of compounds identified and their relative quantities can serve as a basis for comparing normal animals with diseased rats. Since myoinositol can be identified, diabetic rat urine can be investigated in greater detail. The use of reversed-phase HPLC obviates the need to prepare derivatives of the carbohydrates, and hence the actual mono-, di- and polysaccharides present in the urine can be detected. Less time is required for this method, and it is simpler than anion-exchange high-resolution column chromatography used in the automatic analysis of carbohydrates. We are in agreement with Linden and Lawhead[10] that HPLC has advantages over GLC for saccharide determinations.

RESULTS AND DISCUSSION

In the preparation of the rat urine for the HPLC analysis, it was necessary to remove the protein. It was possible to estimate the protein concentration in the urine by the use of the TS meter. By reading the values on the plasma–serum scale and using the conversion table, a value was obtained that corresponded to a refractive index of 1.3612. Since the refractive index and total-solid content are closely correlated, it was possible to extrapolate from the highest value for total solids given for the urine, 10.2 per 100 ml at a refractive index of 1.3488, to 18.5 g total solid per 100 ml at a refractive index of 1.3612. The protein concentration of the urine equals the total-solid concentration of the urine before dialysis minus the total-solid concentration of an equal volume of the dialysate, *i.e.* 18.5 — 5.85 = 12.65 g per 100 ml. Although protein comes off the column before water, protein can form films and clog syringes and tubing; protein also increases the pressure required to force the solvent through the column. Thus, protein should be removed from the urine sample, either by dialysis or by ultra-filtration.

We found that the volume of urine excreted daily by laboratory rats varies greatly. The concentration of solids was higher in the lower volumes and less in the larger volumes. Therefore, mg of carbohydrate per ml could not be used for comparing amounts of various carbohydrates found in rats, whereas the percentage of each carbohydrate of the total non-protein solids served as a good basis for comparison.

Quantitation of the individual saccharides was easily achieved by correlating the peak height with the concentration on a calibration curve using urea, polyol and carbohydrate standards. The latter were made by dissolving a definite amount in 1 ml of water. Injecting samples with varying volumes of standards from 1 μl to 25 ml furnishes enough points to establish a calibration curve. Another method used to obtain points was to establish the calibration curve by measuring the peak height at the original concentration and to repeat after successive dilutions, keeping the injection volume constant. The following amounts of substances were found convenient for each primary standard (before dilution): urea, glycerol and L-arabinose, 20–30 mg/ml; other carbohydrates, including myoinositol, 5 mg/ml.

The substances identified are listed in order of their appearance in Tables II and III. Table IV indicates the amount present of each substance identified.

TABLE II

ELUTION TIME OF UREA, POLYOLS AND SACCHARIDES IN WATER–ACETONITRILE (15:85) AT A FLOW-RATE OF 2.0 ml/min ON A μBONDAPAK-CARBOHYDRATE COLUMN

Substance	*Elution time (min)*	*Substance*	*Elution time (min)*
Urea	2.7	Glucose	7.7
Glycerol	3.4	Galactose	8.4
L-Rhamnose	3.6	Mannoheptulose	9.0
Ribose	4.0	Glucoheptose	10.4
Xylose	4.3	Sucrose	15.0
Fucose	4.4	Myoinositol	18.0
L-Arabinose	5.1	Maltose	19.4
Deoxygalactose	5.5	Lactose	23.9
Fructose	6.3	Melibiose	30.5
Mannose	7.1	Raffinose	43.8

TABLE III

ELUTION TIME OF MYOINOSITOL AND SACCHARIDES IN WATER–ACETONITRILE (25:75) AT A FLOW-RATE OF 2.0 ml/min ON A μBONDAPAK-CARBOHYDRATE COLUMN

Substance	*Elution time (min)*
Sucrose	7.0
Myoinositol	8.7
Maltose	8.9
Lactose	9.4
Melibiose	10.5
Raffinose	14.8

TABLE IV

IDENTIFICATION OF THE QUANTITY OF EACH SUBSTANCE

Substance	*Dialysate (mg/ml)*	*Total non-protein solids (%)*
Urea	22.50	38.5
Glycerol	6.13	10.5
Arabinose	8.95	15.3
Rhamnose	2.40	4.1
Ribose	3.40	5.8
Xylose	1.60	2.7
Fucose	0.73	1.2
Deoxygalactose	1.10	1.8
Fructose	0.35	0.6
Mannose	0.32	0.6
Glucose	0.13	0.2
Galactose	0.25	0.4
Mannoheptulose	0.49	0.8
Glucoheptose	3.90	6.6
Sucrose	0.13	0.3
Myoinositol	0.30	0.5
Maltose	0.38	0.7
Lactose	0.59	1.0
Melibiose	0.55	1.0
Raffinose	0.67	1.2

ACKNOWLEDGEMENT

We should like to thank the Lilly Drake Foundation for Cancer Research for supporting this project.

REFERENCES

1 E. Pitkanen, *Clin. Chim. Acta*, 38 (1972) 221.
2 H. Haga, T. Imanari and Z. Tamura, *Chem. Pharm. Bull.*, 20 (1972) 1805.
3 H. Nakamura and Z. Tamura, *Chem. Pharm. Bull.*, 18 (1970) 2314.
4 H. Nakamura and Z. Tamura, *Chem. Pharm. Bull.*, 20 (1972) 1070.
5 A. Lundblad and S. Svensson, *Biochemistry*, 1 (1972) 306.
6 R. L. Jolley and M. L. Freeman, *Clin. Chem.*, 14 (1968) 538.
7 R. L. Jolley, K. S. Warren, C. D. Scott, J. L. Jainchill and M. L. Freeman. *Amer. J. Clin. Pathol.*, 53 (1970) 701.
8 S. Katz, S. R. Dinsmore and W. W. Pitt, Jr., *Clin. Chem.*, 17 (1971) 731.
9 J. K. Palmer, *Anal. Lett.*, 8 (1975) 215.
10 J. C. Linden and C. L. Lawhead, *J. Chromatogr.*, 105 (1975) 125.