Journal of Chromatography, 166 (1978) 201–212

CHROM. 11,279

ANALYSIS OF MONO- AND DISACCHARIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE BENZYLOXIME-PERBENZOYL DERIVATIVES

RICHARD M. THOMPSON

Department of Pediatrics, Indiana University School of Medicine, Indianapolis, Ind. 46202 (U.S.A.)

(Received May 11th, 1978)

SUMMARY

A group of biologically important mono- and disaccharides are separated by high-performance liquid chromatography of the benzyloxime-perbenzoyl derivatives on a normal-phase microparticulate column with a hexane–dioxane mixture as the eluting solvent. A single, quantifiable derivative of each sugar is formed easily. These derivatives are detected by UV absorption at either 230 or 254 nm, with a sensitivity in the picomole range at the former wavelength. The sugars in the residues from the evaporation of small aliquots of biologic fluids (10–100 μl) are derivatized without prior isolation and are determined quantitatively by the use of appropriate internal standards. The analyses could be performed routinely with a simple, inexpensive instrument.

INTRODUCTION

A wide variety of chromatographic methods for the analysis of mono-, di-, and trisaccharides have been reported previously. One of the major problems with the use of gas chromatography (GC) is that sugars must be derivatized to increase volatility, leading in most cases to multiple products for each due to anomerization or to the formation of both furanose and pyranose rings. In order to circumvent this problem, acyclic oximes[1], nitriles[2], and alditols[3] have been prepared prior to derivatization of the hydroxyl groups to either the acetates or trimethylsilyl ethers, thereby forming a single, asymmetric derivative for each sugar. The GC and GC–mass spectrometric properties of a number of these acyclic products have been well documented[1,3,4].

Most of the techniques currently available for the high-performance liquid chromatographic (HPLC) determination of sugars utilize refractive index detection and are relatively insensitive or require a rather complex apparatus including a post-column reaction mechanism to produce derivatives which can be detected by absorption at an appropriate wavelength[5–7]. These methods may require an elution time of several hours, but usually give single peaks for each saccharide in the biologic fluid under examination.

A recent communication by Lehrfeld[8] describes the HPLC determination of perbenzoylated carbohydrates which can be detected easily by UV absorption. As with the derivatives usually prepared for GC analysis, multiple products are formed during the benzoylation reaction from those sugars having free anomeric centers. The nature of each of the product mixtures (*i.e.*, elution time, number, and relative intensities of the peaks) is characteristic for each sugar, however, and can be used for identification. Indeed, such a procedure is now being utilized in this laboratory for the characterization of reducing substances in urine samples from pediatric patients who may have one of several disorders of carbohydrate metabolism[9].

In order to simplify the determination of sugars in biologic fluids and to provide a means by which they could be quantified easily, the formation and chromatographic properties of the benzyloxime-perbenzoyl (BO, PhCO) derivatives have been examined. As with oxime formation for GC analysis, a single, acyclic derivative is produced from each mono- and disaccharide; these compounds can be detected with a high degree of sensitivity by UV absorption.

EXPERIMENTAL

All standard sugars and benzyloxyamine hydrochloride were from Sigma (St. Louis, Mo., U.S.A.). Pyridine was Eastman (Rochester, N.Y., U.S.A.) spectro-grade, benzoyl chloride was from Aldrich (Milwaukee, Wisc., U.S.A.), and the HPLC-grade solvents were from either Fisher Scientific (Pittsburgh, Pa, U.S.A.) or Burdick and Jackson (Muskegon, Mich., U.S.A.). All other solvents were of reagent grade purity.

Benzyloxime-perbenzoyl derivatives

Fifty to 200 μl of a solution of benzyloxyamine hydrochloride in pyridine (20 mg/ml) (large excess) was added to 100 μg of the standard sugars either individually or in mixtures in small screw-capped vials and the oximation reaction carried out at 60°. After 30 min 200 μl of benzoyl chloride was added; this second reaction was also effected at 60° for 30 min. Excess benzoyl chloride was hydrolyzed with 1–1.5 ml of 80% methanol saturated with sodium carbonate. The derivatives were extracted into hexane (1–1.5 ml), and this fraction was washed once with Na_2CO_3-saturated 80% methanol and once with 80% methanol. Centrifugation was used when necessary to completely separate the hexane and 80% methanol layers. The washed hexane extract was evaporated to dryness under nitrogen. The residue was redissolved in HPLC-grade hexane for chromatographic analysis.

Small aliquots of biologic fluids (50–100 μl), including appropriate internal standards, were evaporated to dryness under nitrogen. The residual sugars were derivatized as above.

Perbenzoyl derivatives were formed simply by carrying out the second step of the above procedure.

High-performance liquid chromatography

The analytical separations were carried out with a Waters Assoc. component instrument consisting of a U6K injector, two Model 6000 solvent delivery systems, a Model 660 solvent programmer, and a Model 450 variable-wavelength detector. The system was used in the dual-pump gradient elution mode for development of

the procedure, then in the single-pump isocratic mode for the routine analyses. Operating parameters included: Waters (Milford, Mass., U.S.A.) μPorasil column; eluting solvent, hexane–dioxane (80:20); flow-rate, 1.0 ml/min; injection volume 10–20 μl; detector at 230 or 254 nm, 0.02–0.4 a.u.f.s.; and recorder speed, 5 min/in.

Standard curves

Standard curves for the quantification of certain sugars were established by adding varying amounts (0–1000 μg) of each in duplicate along with an internal standard such as xylitol (80 μg per 100 μl) or mannitol (100 μg per 100 μl) to 100 μl of water or serum, evaporating to dryness, and forming the BO, PhCO derivatives of the residues. After chromatography, peak height ratios were determined and plotted *versus* sugar concentration. Linear regression equations and coefficients were calculated by standard methods.

RESULTS

Figs. 1a, b, and c and 2a, b, and c show the chromatograms obtained for the BO, PhCO derivatives of three pentoses and three hexoses, respectively. Each monosaccharide is eluted as a single sharp peak in the system described above. The

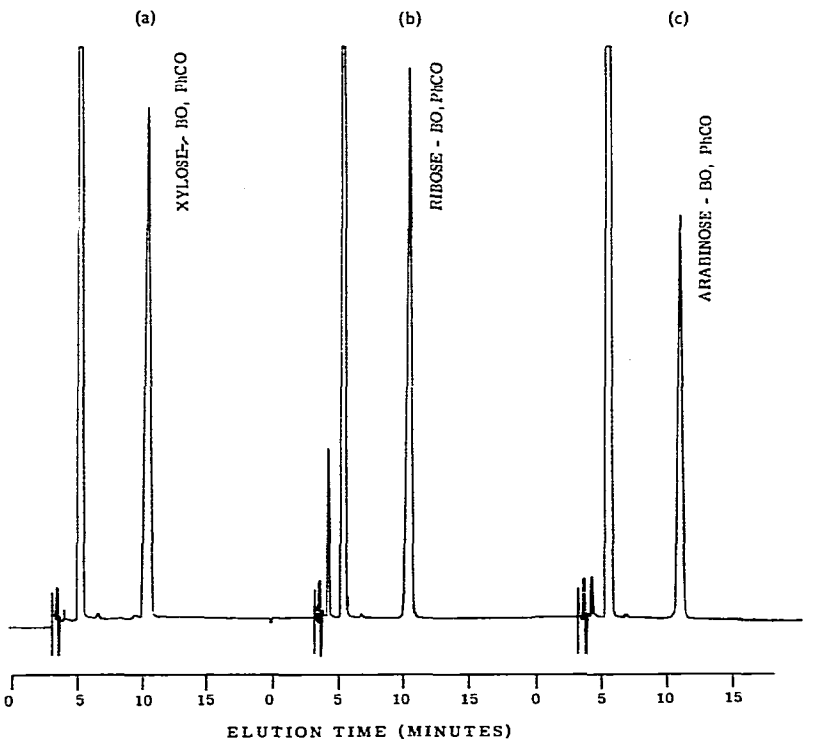


Fig. 1. (a) HPLC chromatogram of the BO, PhCO derivative of xylose. Conditions: Waters μPorasil column; hexane–dioxane (80:20); flow-rate 1.0 ml/min; detection at 230 nm. (b) HPLC chromatogram of the BO, PhCO derivative of ribose. Conditions same as in Fig. 1a. (c) HPLC chromatogram of the BO, PhCO derivative of arabinose. Conditions same as in Fig. 1a.

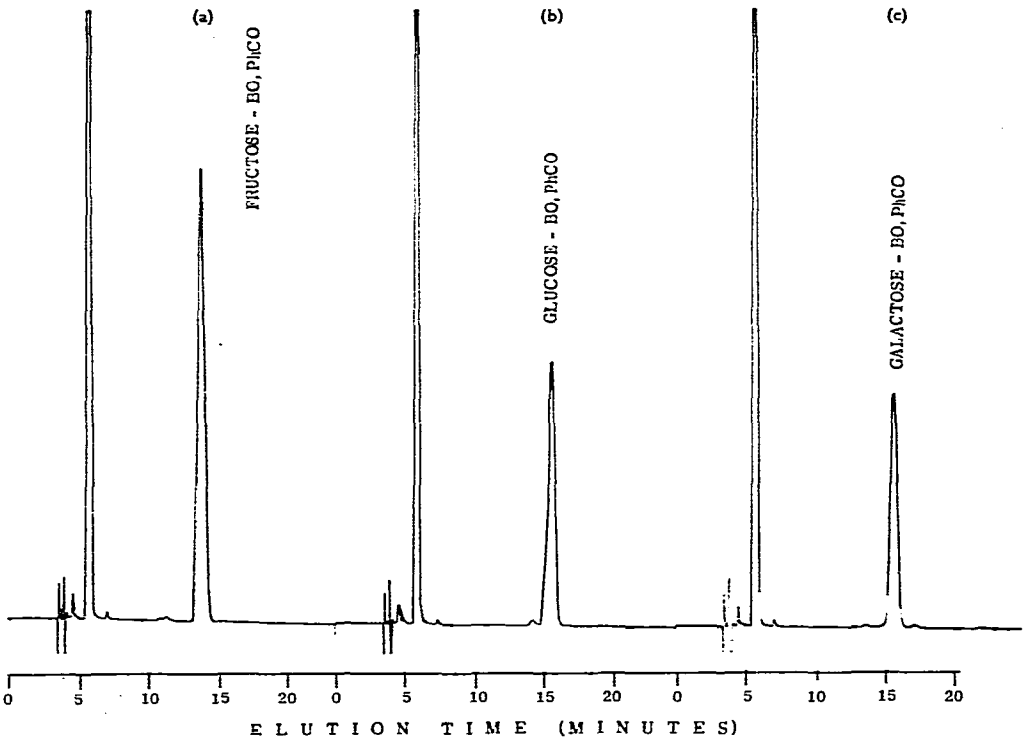
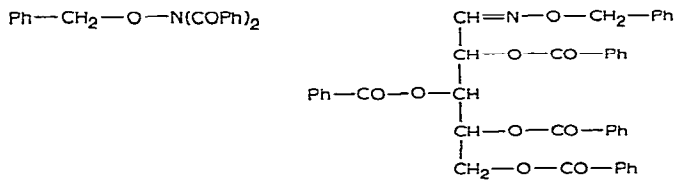


Fig. 2. (a) HPLC chromatogram of the BO, PhCO derivative of fructose. Conditions same as in Fig. 1a. (b) HPLC chromatogram of the BO, PhCO derivative of glucose. Conditions same as in Fig. 1a. (c) HPLC chromatogram of the BO, PhCO derivative of galactose. Conditions same as in Fig. 1a.

second earlier eluting component common to all chromatograms is probably the N,N-dibenzoyl derivative of benzyloxyamine (I), a volatile compound which was characterized by GC–mass spectrometry (Table I).



(I) (II)

TABLE I

PARTIAL 70 eV MASS SPECTRA OF COMPOUNDS I AND II*

Compound	*m/e (% relative intensity)*
N,N-Dibenzoylbenzyloxyamine (I)	77 (82), 91 (81), 105 (100), 122 (12), 197 (13), 210 (10), 224 (5.0), 225 (8.6), 331 (35).
2,3,4,5-Tetra-O-benzoylxylose benzyloxime (II)	77 (39), 91 (72), 105 (100), 122 (10), 195 (2.0), 216 (2.1), 294 (2.5), 403 (1.0), 459 (27), 549 (0.4), 550 (0.3), 671 (0.4).

* LKB-9000-S instrument.

In order to verify the structure of the BO, PhCO derivatives, the mass spectrum of the BO-tetrabenzoyl derivative of xylose (II) was obtained (Table I). The molecular weight (671 a.m.u.) is correct, and most of the fragments can be explained as occurring by standard processes.

The derivatives of three disaccharides —sucrose (PhCO), maltose (BO, PhCO), and lactose (BO, PhCO)— are represented in Figs. 3, 4, and 5. Since sucrose does not have a free anomeric center, it does not form a benzyloxime and is, therefore, determined as the octabenzoyl derivative. Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), considered to be a possible internal standard, also yields only the octabenzoyl derivative, but this compound cochromatographs with the BO, PhCO derivatives of lactose and maltose and is not shown here. Both maltose and lactose do form benzyloximes prior to perbenzoylation.

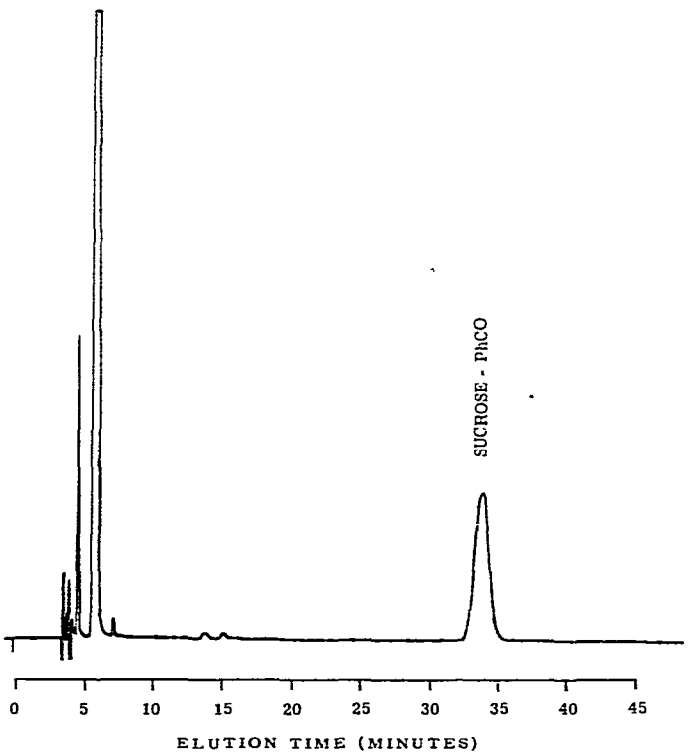


Fig. 3. HPLC chromatogram of the PhCO derivative of sucrose. Conditions same as in Fig. 1a.

Figs. 6a and 6b show the separation of the mixture of these nine sugars under the conditions described above and detected at 230 nm and 254 nm, respectively. A loss of approximately 95% in sensitivity was observed at the latter wavelength, since 230 nm is near the UV maximum for these derivatives. Mannitol is present (PhCO derivative) as a possible internal standard. The potential sensitivity of this analytical technique is demonstrated in Fig. 7. The small peak indicated by the arrow represents an injection of the BO, PhCO derivative of 2 ng of ribose detected at 230 nm and 0.02 a.u.f.s.

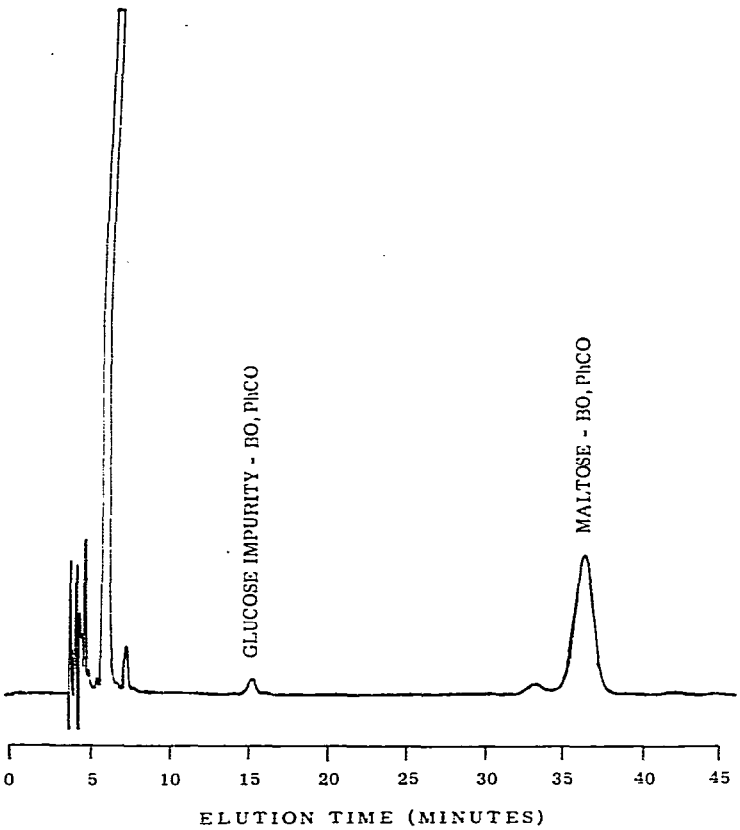


Fig. 4. HPLC chromatogram of the BO, PhCO derivative of maltose. Conditions same as in Fig. 1a.

As an example of the capability of this method for quantification of a sugar in a biologic fluid, Figs. 8 and 9 (a and b) contain the standard curve obtained for glucose with xylitol as the internal standard and two typical chromatograms (230 nm, 0.4 a.u.f.s.) of derivatized control sera containing two different concentrations of glucose. The standard curve is linear over a wide range, the linear regression equation being $y = 0.009\,x - 0.0005$ and the regression coefficient, $r = 0.9999$. The values for glucose in Figs. 8a and 8b were determined to be 89.7 mg/dl and 170.7 mg/dl, respectively. By comparison, a routine procedure utilizing glucose oxidase yielded 86 mg/dl and 168 mg/dl for the same serum samples. Similar chromatograms were obtained at 254 nm and 0.04 a.u.f.s.

DISCUSSION

The major purpose of this effort was to study the properties of a derivative which would be suitable for the quantification of sugars in biologic mixtures by HPLC. The previously described perbenzoyl derivative[8] is useful for the identification of carbohydrates by HPLC, but cannot be utilized conveniently for quantitative analysis because of the formation of multiple products from most sugars.

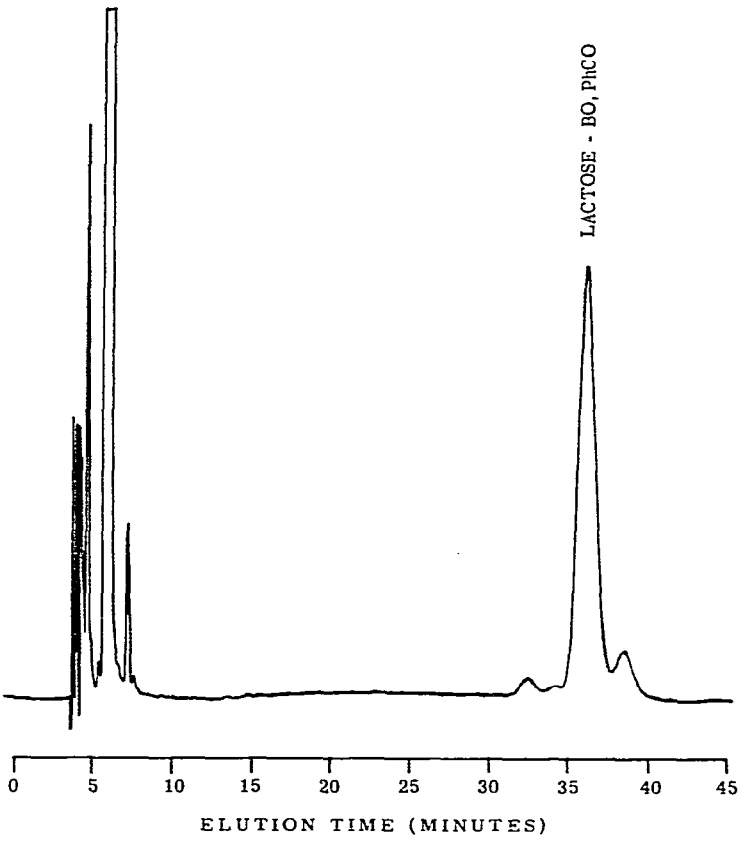


Fig. 5. HPLC chromatogram of the BO, PhCO derivative of lactose. Conditions same as in Fig. 1a.

Since oximation had been used in the past in order to form single derivatives for the GC analysis of sugars[1], oxime-PhCO, methoxime-PhCO, and BO, PhCO derivatives of glucose and galactose were prepared and examined by HPLC on the normal-phase microparticulate column with various hexane–dioxane mixtures as the eluting solvents. The oxime-PhCO derivatives of these monosaccharides were not single products, possibly due to small amounts of dehydration to yield the acyclic nitriles[2].

Both the methoxime-PhCO and BO, PhCO derivatives were single products and had similar chromatographic properties. The latter were chosen for further study because of the potential enhancement in detectability due to the extra phenyl group. Indeed, these derivatives can be detected at 230 nm down to the 10-pmole level (Fig. 7). The chromatograms in Fig. 9 represent injections of the equivalent of approximately 700 nl of serum (630–1120 ng of glucose). If the detector were monitoring at 0.04 a.u.f.s., these injection volumes could be reduced tenfold. At 254 nm and 0.04 a.u.f.s., however, at least the former amount must be injected, a reasonable compromise so that the total method may be performed with a single-wavelength detector. It may be possible to increase this sensitivity further by the use of the *p*-

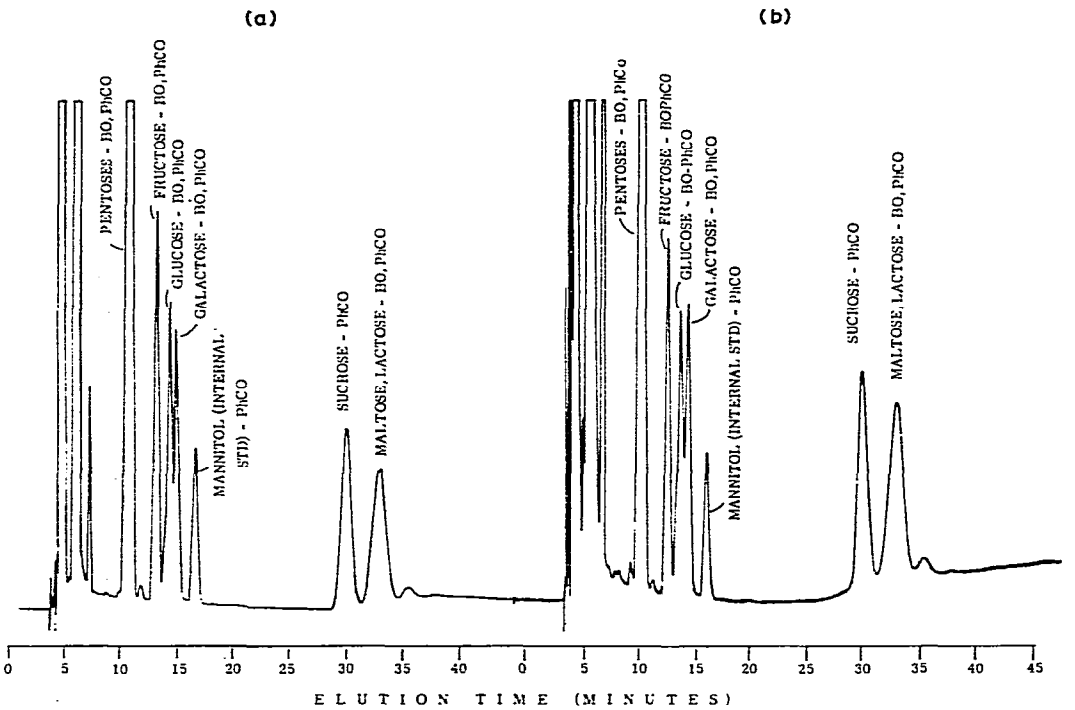
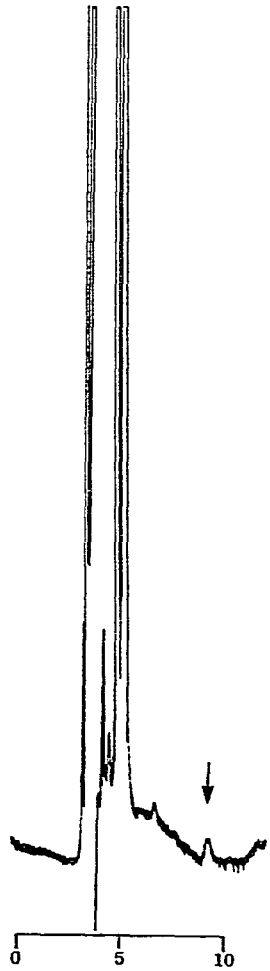


Fig. 6. (a) HPLC separation of the BO, PhCO derivatives of nine mono- and disaccharides. Conditions same as in Fig. 1a, 0.4 a.u.f.s. (b) HPLC separation of the BO, PhCO derivatives of nine mono- and disaccharides. Conditions same as in Fig. 1a, except detection at 254 nm, 0.04 a.u.f.s.

nitrobenzyloxime-3,5-dinitrobenzoyl derivatives formed from the appropriate reagents.

The normal-phase Waters μPorasil column and a dioxane–hexane eluting solvent serve as an excellent combination for the HPLC analysis of the BO, PhCO derivatives of mono- and disaccharides. After more than 1000 injections on a single column, there have been essentially no changes in peak shape, retention times, or column pressure (approx. 200 p.s.i.). Early attempts to separate these derivatives on a reversed-phase Waters μBondapak column with acetonitrile–water and methanol–water combinations as the eluting solvents were not so successful, because the peaks were broader and usually not so well resolved.

It is obvious that all of the sugars in the chromatograms in Fig. 6 could not be quantified if all were present in a mixture under analysis. The BO, PhCO derivatives of the three pentoses —ribose, xylose, and arabinose— are clearly not separated, nor are they separable with lower concentrations of dioxane. Therefore, only a value for total pentose could be calculated if two or more were present; this value is the same as is determined colorimetrically by various procedures used in the routine clinical laboratory[10,11]. However, if only one pentose appears to a significant extent in the sugar mixture, such as xylose in serum during a xylose test for intestinal absorption, then it could be quantified accurately and without interference.



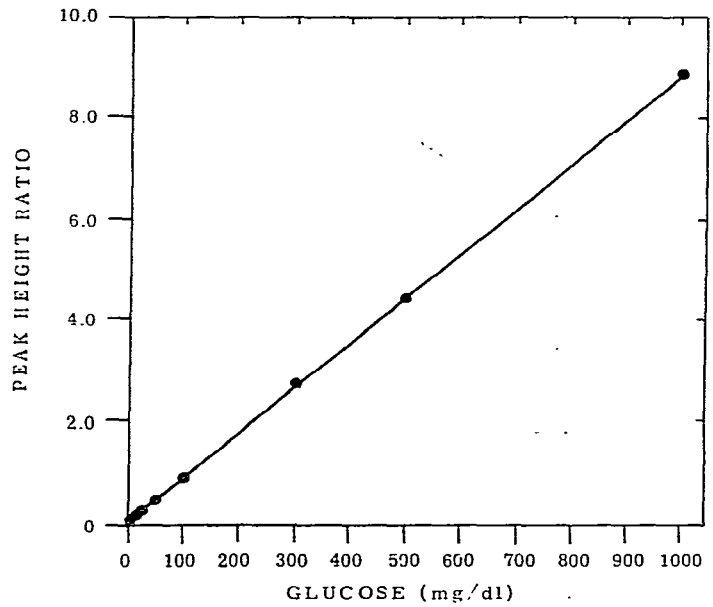


Fig. 7. HPLC response from 2 ng of ribose as the BO, PhCO derivative. Conditions same as in Fig. 1a, 0.02 a.u.f.s.

Fig. 8. Standard curve for the quantification of glucose. The internal standard was xylitol at a concentration of 80 mg/dl.

The three hexoses of major clinical interest —fructose, glucose, and galactose— are much better resolved than the pentoses. Fructose could be quantified in the presence of any of the other sugars by either peak height or peak area measurements; glucose and galactose could be determined by instrumental integration, although these latter two sugars are separated completely with an eluting solvent of lower polarity such as hexane–dioxane (85:15). Either xylitol (shown in Fig. 9) or mannitol (Fig. 6) is an excellent internal standard for the determination of these monosaccharides if it is not present in the sugar mixture.

The measurement of disaccharides is of clinical interest especially when

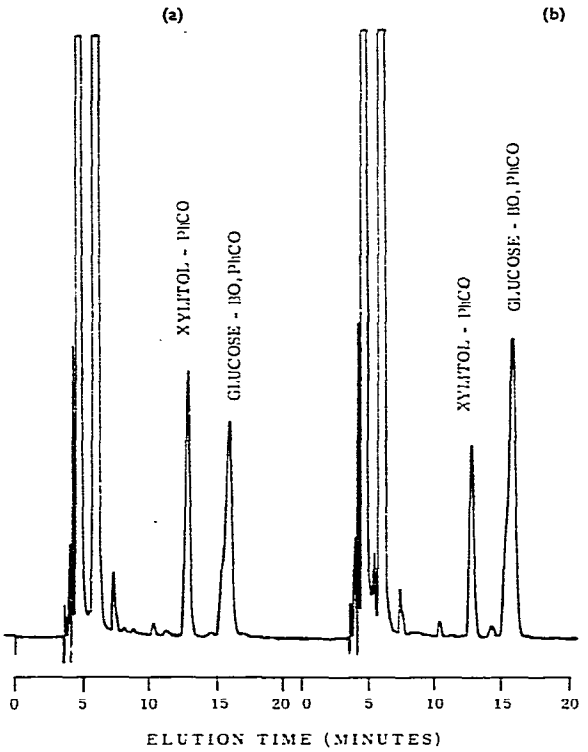


Fig. 9. (a) HPLC separation of the BO, PhCO derivatives of the sugars in a control serum specimen. Conditions same as in Fig. 1a. Glucose concentration by HPLC = 89.7 mg/dl; by glucose oxidase = 86 mg/dl. (b) HPLC separation of the BO, PhCO derivatives of the sugars in a control serum specimen. Conditions same as in Fig. 1a. Glucose concentration by HPLC = 170.7 mg/dl; by glucose oxidase = 168 mg/dl.

screening patients for one of several intestinal disaccharidase deficiencies. Since the octabenzoyl derivative of sucrose is cleanly separated from the other disaccharides in this system, sucrose could be determined easily with mannitol as the internal standard. As with the pentoses, only a total lactose–maltose value could be calculated, although it is unlikely that both of these disaccharides would occur to a significant extent in the same mixture. It is unfortunate that the octabenzoyl derivative of trehalose cochromatographs with the BO, PhCO derivatives of lactose and maltose, because the former would be an excellent internal standard. On the other hand, if the PhCO derivatives of a mixture are prepared, sucrose and trehalose are resolved completely from each other and from both anomers of lactose and maltose[9]. Under these conditions, trehalose can be used for the accurate measurement of sucrose.

Forming derivatives of sugars in the residues obtained from the evaporation of small aliquots of a biologic fluid is merely an extension of the previously described procedures for the GC determination of sugars and amino acids in urine[12], drugs, drug metabolites, and other compounds in bile[13] and urine[14], and disaccharides in feces[15]. The reaction proceeds cleanly and quantitatively, as evidenced

by the excellent correlation between the values obtained for glucose in control serum by both this HPLC method and a routine specific procedure using glucose oxidase (Fig. 9). Other fluids which have been studied include saliva, semen, feces, urine, and amniotic, cerebrospinal, and synovial fluids[9].

The linearity of the standard curves for various sugars established over a wide concentration range by the HPLC technique is excellent, as exemplified by the standard curve for glucose in Fig. 8. Although this curve was determined in aqueous media, not in a biologic fluid, the accuracy with which glucose in serum can be calculated by the regression equation above is remarkable. Because the standard curve is linear over a wide range, the usefulness of this assay is not limited to the analysis of mono- and disaccharides in biologic fluids, but can also be extended to other mixtures such as hydrolysates of polysaccharides or foodstuffs.

In conclusion, a rapid, sensitive, and relatively simple HPLC method has been developed for the identification and quantification of carbohydrates in biologic fluids. The advantages of this procedure over those published previously include a faster analysis time, sensitivity to the nanogram level, use of very small amounts of biologic fluids, and the lack of a requirement for other than a basic inexpensive commercially available instrument consisting of a single pump for isocratic elution, a normal-phase microparticulate column, a standard ultraviolet detector to monitor the effluent at 254 nm, and a recorder. Although a greater ability to detect the BO, PhCO derivatives is observed at 230 nm with a variable-wavelength detector, the loss in sensitivity at 254 nm is not sufficient to be a problem unless one wishes to detect less than 100 ng. Therefore, the technique described above can be easily instituted as a routine laboratory procedure either to screen for disorders of carbohydrate metabolism[9] or to determine those sugars which are difficult to quantify by other methods.

ACKNOWLEDGEMENTS

This research was supported in part by a grant from the Public Health Service (NIH AM-17,665). The author is also indebted to Dr. Melvin R. Glick and Mr. Richard B. Sample of the Department of Clinical Pathology for providing the serum specimens and carrying out the glucose oxidase assays and statistical calculations.

REFERENCES

1 R. A. Laine and C. C. Sweeley, *Carbohyd. Res.*, 27 (1973) 199.
2 C. D. Pfaffenberger, J. Szafranek, M. G. Horning and E. C. Horning, *Anal. Biochem.*, 63 (1975) 501.
3 J. Kärkkäinen, *Carbohyd. Res.*, 11 (1969) 247.
4 J. Szafranek, C. D. Pfaffenberger and E. C. Horning, *Carbohyd. Res.*, 38 (1974) 97.
5 R. L. Jolley and M. L. Freeman, *Clin. Chem.*, 14 (1968) 538.
6 R. L. Jolley, K. S. Warren, C. D. Scott, J. L. Jainchill and M. L. Freeman, *Amer. J. Clin. Pathol.*, 53 (1970) 793.
7 R. Seuffer, W. Voelter and H. Bauer, *J. Clin. Chem. Clin. Biochem.*, 15 (1977) 663.
8 J. Lehrfeld, *J. Chromatogr.*, 120 (1976) 141.
9 R. M. Thompson, unpublished results.
10 J. H. Roe and E. W. Rice, *J. Biol. Chem.*, 173 (1948) 507.

11 P. Trinder, *Analyst (London)*, 100 (1975) 12.
12 E. Jellum, O. Stokke and L. Eldjarn, *Clin. Chem.*, 18 (1972) 800.
13 R. M. Thompson, N. Gerber, R. A. Seibert and D. M. Desiderio, *Drug Metab. Disp.*, 1 (1973) 489.
14 R. M. Thompson, *Res. Commun. Chem. Pathol. Pharmacol.*, 16 (1977) 145.
15 R. M. Thompson, R. D. Coffin, M. R. Glick and J. F. Fitzgerald, *Clin. Chim. Acta*, 84 (1978) 185.