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SEPARATION OF CARBOHYDRATES ON A NEW POLAR BONDED PHASE MATERIAL

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SUMMARY

The separation of a number of carbohydrates on Partisil-10 PAC, a 10-μm silica gel with a permanently bonded polar phase, is described. The basic mobile phase is acetonitrile–water, but various acids or salts may be added to the aqueous portion to change the selectivity of the separation. A study of the effect of H_3PO_4, HCl, $HClO_4$, KH_2PO_4, and sodium acetate on the separation of mono-, di-, and trisaccharides is discussed and chromatograms of several real sample solutions are shown.

INTRODUCTION

For researchers involved in biochemistry or in the food industry, carbohydrate analysis continues to grow in importance. Previous analysis of this class of compounds was slow and quite unsatisfactory. Such separations were performed on cellulose or ion-exchange resins and frequently required detection by colorimetric derivatization after chromatography[1]. The most recent work with ion-exchange resins has involved the use of various counter-ions and elution with water or ethanol–water to accomplish partition separations of carbohydrates[2–6]. Fortunately for the analytical chemist of today, recent developments in the technology of packings for high-performance liquid chromatography (HPLC) have led to faster and more selective media for the carbohydrate separation. In the past two years researchers have been reporting on carbohydrate separations on these new, stable, siloxane-bonded media for HPLC[7–9]. Aside from a recent paper by Schwarzenbach[10], in which a 3-aminopropyl-bonded phase gave carbohydrate separations, very little information has been reported on the chemistry of these new packings or the mechanism of the separation. Indeed, reference is made to "reversed-phase partition" chromatography in such separations when, actually, normal-phase partition chromatography appears to be the mode of separation[9].

Because of the selectivity changes possible by varying the mobile phase, only one polar bonded phase is necessary for normal-phase partition chromatography. Historically, the most useful polar bonded phases have been ether, cyano, and amino; so, a complex bonded phase which would give the best features of each was developed. The product was named Partisil-10 PAC and is a most versatile packing for normal

partition LC. Much work is being done on this stationary phase to separate polar solutes by eluting with heptane modified with ethanol, acetonitrile, or chloroform. This study deals, however, with the novel separations possible on Partisil-10 PAC of a number of carbohydrates using combinations of acetonitrile and water as eluent.

EXPERIMENTAL

Apparatus and material

The liquid chromatograph used for this study consisted of the following components: a Varian Model 4000 cylinder pump (Palo Alto, Calif., U.S.A.); a Whatman LIC syringe septum injector (Clifton, N.J., U.S.A.); a Hamilton No. 702 (25-μl) syringe (Reno, Nev., U.S.A.); a Whatman Partisil-10 PAC PXS 10/25 column; a Laboratory Data Control refractive index detector (Riviera Beach, Fla., U.S.A.); and a Varian Model A-25 recorder.

The carbohydrate chromatographic standards were purchased from ICN Nutritional Biochemicals (Cleveland, Ohio, U.S.A.). Acetonitrile was distilled-in-glass, manufactured by Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). The salts and acids were from J. T. Baker (Phillipsburg, N.J., U.S.A.). The maple sugar sample was obtained from Laboratoire de Chimie, Complexe Scientifique (Ste. Foy, Quebec, Canada). The soybean extract was donated by Professor P. A. Sieb, Kansas State University. Hycal® is a product of Beecham Labs., Brentford, Great Britain. Coca-Cola® is a product of the Coca-Cola Bottling Co., Atlanta, Ga., U.S.A. Sweet 'n Low® is a product of the Cumberland Packing Corp., Brooklyn, N.Y., U.S.A. All other foodstuffs were obtained from local stores.

Preparation of the Partisil-10 PAC columns

The Partisil-10 PAC packing is made by reacting various organochlorosilanes with Partisil-10, but the details of this reaction are proprietary. To prepare properly packed columns, Partisil-10 PAC is slurried with chloroform–methanol (4:1) (1 g packing/10 ml) and pumped at 10,000 p.s.i. from a reservoir into a 25 cm × 1/4 in. O.D. × 4.6 mm I.D. seamless, smooth-bore, 316 stainless-steel column. The column fittings are zero dead volume (ZDV) fittings containing a removable 1/4-in. diameter, 2-μm porosity, 316 stainless-steel frit. Only columns reaching a minimum efficiency of 15,000 plates/m ($H = 0.067$ mm) with a standard test mixture of carbon tetrachloride, benzene, and naphthalene with a mobile phase of heptane are used in this study. All Partisil-10 PAC columns are activated with ethanol, acetone, ethyl acetate, 1,1,1-trichloroethane, and heptane before testing. Details of the drying procedure are given elsewhere[11].

RESULTS AND DISCUSSION

Initial separations of carbohydrate samples on Partisil-10 PAC showed poor separations of components with methanol–water mobile phases. It was considered that the less polar character of acetonitrile might be a better solvator of the bonded phase and allow greater selectivity. Acetonitrile–water combinations, indeed, did have much better selectivity, but with the disadvantage of peak tailing. Phosphoric acid was added to the mobile phase to bring its pH to 5.0, which eliminated much,

and sometimes all, of the tailing. Fig. 1 shows a variety of mono- and disaccharides separated with this mobile phase. High-molecular-weight saccharides were eluted by increasing the water content of the mobile phase (Fig. 2 and Table I), suggesting separation by normal-phase partition chromatography rather than reversed-phase partition chromatography. At any given mobile phase composition, a plot of k' *versus* chain-length of the carbohydrate produces a straight line. A similar relationship was reported by Havlicek and Samuelson[3], who also found the slope to be related to both the concentration of ethanol in the mobile phase and the type of glycosidic linkage.

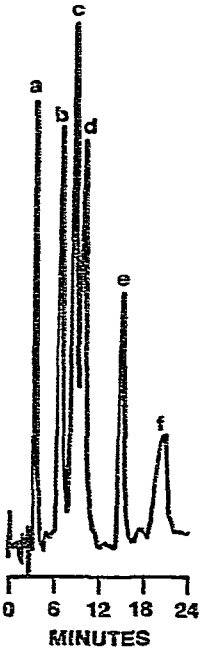

Fig. 1. Separation of mono- and disaccharides. Column, 25 cm × 4.6 mm I.D., Partisil-10 PAC PXS 10/25; mobile phase, acetonitrile–water (80:20), pH 5.0 with H_3PO_4; flow-rate, 1.3 ml/min; pressure, 350 p.s.i.; detection, refractive index, × 0.04. a = Solvent front; b = xylose; c = fructose; d = glucose; e = sucrose; f = lactose.

Because of the complex structure of the Partisil-10 PAC bonded phase, the effect of varying pH on the selectivity for the carbohydrate separation was studied. Table II shows the k' values of the carbohydrates when making pH changes on Partisil-10 PAC using phosphoric acid and potassium hydroxide. No apparent trend in the change of k' with pH was noted except that at a pH of 5.0, a minimum k' value was frequently encountered. The general peak shape was rated on a relative scale from excellent to poor (Table II) and was found to be best for the largest number of carbohydrates at a pH of 5.0. Accordingly, pH 5.0 is considered optimal and was used exclusively in this study.

Because various anions might interact with the Partisil-10 PAC bonded phase and with the carbohydrates (*i.e.*, borate), the effect of a number of acids and salts at

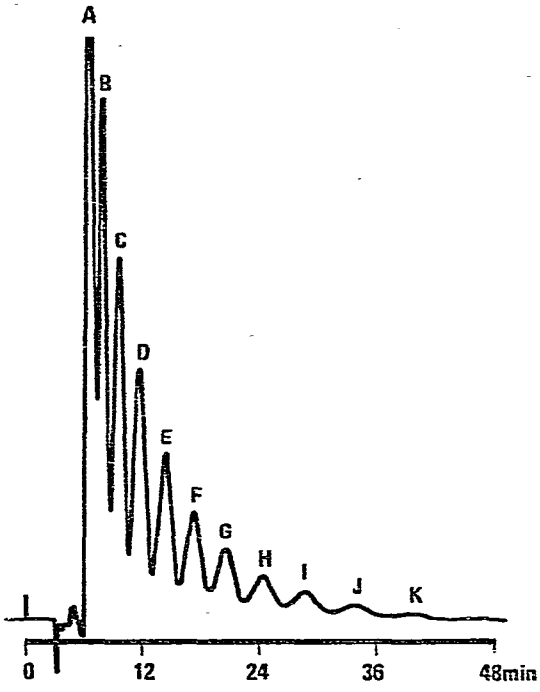

Fig. 2. Separation of oligosaccharides. Column, 250 mm × 4.6 mm I.D., Partisil-10 PAC; mobile phase, acetonitrile–0.0025 *M* $NaC_2H_3O_2$ (65:35), pH 5.0 with acetic acid. Flow-rate, 65 ml/h; pressure, 400 p.s.i.; detection, refractive index, 6.4 × 10^{-4}. Sample: Hycal; 25 μl of pure sample injected. A = Glucose (mono); B = maltose (di); C = trisaccharides; D = tetrasaccharides; E = pentasaccharides; F = hexasaccharides; G–K = higher saccharides.

TABLE I

CAPACITY FACTORS (k') OF OLIGOSACCHARIDES WITH CHANGES IN CONCENTRATION OF ACETONITRILE

All mobile phases are ratios of acetonitrile–0.0025 *M* sodium acetate, pH 5.0 with acetic acid.

Saccharide	*Mobile phase ratio*		
	70:30	*65:35*	*60:40*
Glucose (mono)	0.35	0.30	0.13
Maltose (di)	1.22	0.55	0.50
Trisaccharide	1.97	0.95	0.75
Tetrasaccharide	3.05	1.45	1.00
Pentasaccharide	4.51	1.95	1.25
Hexasaccharide	5.03	2.60	1.50
Heptasaccharide	7.92	3.30	1.88
Octasaccharide	*	4.15	2.13
Nonasaccharide	*	5.05	2.51
Decasaccharide	*	6.20	2.88

* Peaks too broad to measure accurately.

TABLE II

CAPACITY FACTORS (k') OF CARBOHYDRATES WITH CHANGE IN pH

All systems have a mobile phase of acetonitrile–water in the ratio 75:25. Key for peak evaluation: Ex = excellent, VG = very good, G = good, F = fair, P = poor.

Carbohydrate	*pH 3.0 with H_3PO_4*	*pH 5.0 with H_3PO_4*	*pH 6.1 distilled water*	*pH 7.1 with KOH*
Xylose	1.18 Ex	1.16 Ex	1.17 G	1.19 Ex
Arabinose	1.47 G	1.42 G	1.36 G	1.44 G
Ribose	0.94 Ex	0.89 Ex	0.86 Ex	0.88 Ex
Lyxose	1.02 Ex	1.12 Ex	1.06 Ex	1.00 Ex
Fucose	0.94 and 1.22 P	1.02 and 1.22 P	1.00 and 1.24 P	1.00 and 1.24 P
Fructose	1.59 Ex	1.53 Ex	1.42 Ex	1.44 Ex
Mannose	1.49 G	1.58 G	1.61 F	1.59 Ex
Glucose	1.83 F	1.84 G	1.91 F	1.83 F
Galactose	2.06 and 2.35 P	1.93 and 2.26 P	2.00 and 2.35 P	1.83 and 2.16 P
Sucrose	3.18 Ex	2.92 Ex	3.32 Ex	2.81 Ex
Maltose	3.67 P	3.47 G	3.60 P	3.67 P
Lactose	4.11 F	3.86 G	4.26 P	4.11 F
Melibiose	5.22 F	4.05 F	5.47 P	5.00 F
Cellobiose	3.55 F	3.47 G	3.63 P	3.50 F
Raffinose	6.94 G	7.26 VG	7.78 G	7.53 VG

a pH of 5.0 and at fixed ionic strength was investigated. The results of the acid-modified mobile phases are also summarized in Table III. Very small quantities of acid were necessary to adjust the pH (approximately 10^{-6} M). The water is pH adjusted with the acid before mixing with the acetonitrile. Again, no consistent trend with the various acids tested is observed except that nine of the fifteen carbohydrates have their greatest retention with the perchloric acid-modified mobile phase.

The effect of anions from the salts, KH_2PO_4 and sodium acetate, on the separation are noted in Table IV. As with the addition of different acids, these salts change the retention of the carbohydrates. Capacity factors are consistently equal to or less than those obtained with only phosphoric acid added to the mobile phase. In the case of the disaccharides, maltose, and cellobiose, dilute phosphoric acid in the mobile phase gives no separation. With KH_2PO_4 in the mobile phase, cellobiose elutes before maltose; however, with sodium acetate in the mobile phase, maltose elutes before cellobiose.

The most obvious trend, seen in Tables II–IV, is that each type of carbohydrate falls within a specific k' region with any given mobile phase. Martinsson and Samuelson[2] found a similar elution pattern from their partition separations on ion-exchange/ethanol–water systems. Thus, from Table II, the pentoses (xylose, arabinose, ribose, lyxose, fucose) have k' values between 0.94 and 1.47 at a pH of 3; the hexoses (fructose, mannose, glucose, galactose), between 1.49 and 2.35 at a pH of 3; and the disaccharides, between 3.18 and 5.22 at a pH of 3. Similar distinct ranges are noted with all of the mobile phases tried.

Goulding[5] reported the separation of α- and β-anomers of certain carbohydrates in his work. In this study, distinct doublets were noted for fucose and galactose and were attributed to the anomers (see Tables II–IV). Authentic samples

TABLE III

CAPACITY FACTORS (k') OF CARBOHYDRATES WITH CHANGES IN ACID USED TO ADJUST pH

All systems have a mobile phase of acetonitrile–water in the ratio 75:25, pH 5.0. Key for peak evaluation, see Table II.

Carbohydrate	*H_3PO_4*	*$HClO_4$*	*HCl*	*HOAc**	*H_3BO_3**
Xylose	1.16 Ex	1.11 G	1.12 Ex	1.13 Ex	1.07 Ex
Arabinose	1.42 G	1.39 F	1.22 G	1.38 G	1.36 Ex
Ribose	0.89 Ex	0.94 Ex	0.74 Ex	0.86 Ex	0.33 G
Lyxose	1.12 Ex	1.11 Ex	0.98 Ex	1.08 Ex	1.00 VG
Fucose	1.02 and	1.06 and	1.13 and	1.00 and	1.07 G
	1.22 P	1.28 P	1.34 P	1.21 P	
Fructose	1.53 Ex	1.53 Ex	1.44 Ex	1.44 Ex	—
Mannose	1.58 G	1.53 G	1.55 G	1.35 F	1.44 G
Glucose	1.84 G	2.06 F	1.78 F	1.53 Ex	1.54 Ex
Galactose	1.93 and	2.06 and	1.83 and	1.59 and	2.02 VG
	2.26 P	2.26 P	2.17 P	1.76 P	
Sucrose	2.92 Ex	3.06 Ex	3.02 Ex	2.40 Ex	2.80 Ex
Maltose	3.47 G	4.06 F	3.56 F	2.63 Ex	2.82 VG
Lactose	3.86 G	4.28 F	3.89 F	3.06 Ex	3.08 VG
Melibiose	4.05 F	5.28 F	4.89 F	3.55 G	3.47 F
Cellobiose	3.47 G	3.78 F	3.44 F	2.73 Ex	2.59 VG
Raffinose	7.26 VG	7.56 VG	7.06 VG	4.65 Ex	5.30 VG

* Added in proof.

TABLE IV

CAPACITY FACTORS (k') OF CARBOHYDRATES WITH ACETONITRILE WITH VARIOUS BUFFERS

All systems are 75% acetonitrile and 25% buffer. Key for peak evaluation, see Table II.

Carbohydrate	*0.0025 M KH_2PO_4, pH 5.0 with KOH*	*water, pH 5.0 with H_3PO_4*	*0.0025 M $NaC_2H_3O_2$, pH 5.0 with acetic acid*
Xylose	1.00 Ex	1.16 Ex	1.13 Ex
Arabinose	1.22 G	1.42 G	1.26 VG
Ribose	0.74 Ex	0.89 Ex	0.88 Ex
Lyxose	0.94 Ex	1.12 Ex	1.00 Ex
Fucose	0.94 and	1.02 and	0.94 and
	1.11 P	1.22 P	1.15 P
Fructose	1.26 Ex	1.53 Ex	1.40 Ex
Mannose	1.44 G	1.58 G	1.50 VG
Glucose	1.58 G	1.84 G	1.59 Ex
Galactose	1.89 and	1.93 and	1.93 P
	2.22 P	2.26 P	
Sucrose	2.61 Ex	2.92 Ex	2.28 Ex
Maltose	2.89 G	3.47 G	2.94 G
Lactose	3.59 G	3.86 G	3.35 VG
Melibiose	3.89 G	4.05 F	4.00 Ex
Cellobiose	2.60 Ex	3.47 G	3.13 Ex
Raffinose	6.00 Ex	7.26 VG	5.78 Ex

of anomers were not available for confirmatory studies, but other samples of these two carbohydrates exhibited the same doublet, with peak ratios being the same. Results of further work will be reported at a later date.

To demonstrate the range of application available for real carbohydrate samples on Partisil-10 PAC, a number of authentic samples which had been submitted for analysis were separated and their chromatograms given.

Lactose in whole milk is easily and reproducibly determined if the milk is first extracted with carbon tetrachloride to remove lipophilic material and a portion of the aqueous solution injected (Fig. 3). Samples of evaporated milk and low-fat milk gave similar chromatograms without this extraction step.

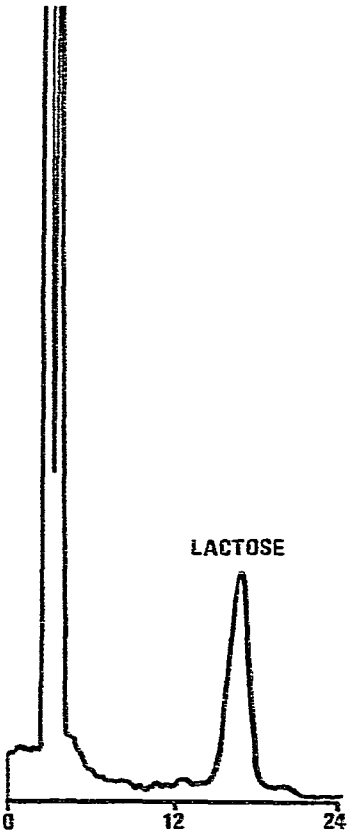

Fig. 3. Separation of lactose in whole milk. Column, 250 mm × 4.6 mm I.D., Partisil-10 PAC; mobile phase, acetonitrile–0.0025 *M* KH_2PO_4 (80:20), pH 5.0 with KOH; flow-rate 90 ml/h; pressure, 300 p.s.i.; detection, refractive index, × 0.02. Sample: Homogenized whole milk; fats removed by carbon tetrachloride extraction.

The quality control of beverages is simple, as exemplified in Fig. 4 by a sample of a cola, indicating the presence of fructose, glucose, and sucrose.

Natural products have to be closely monitored to detect dangerous or illegal adulterants, and the quality control of one such material is seen in Fig. 5, a sample

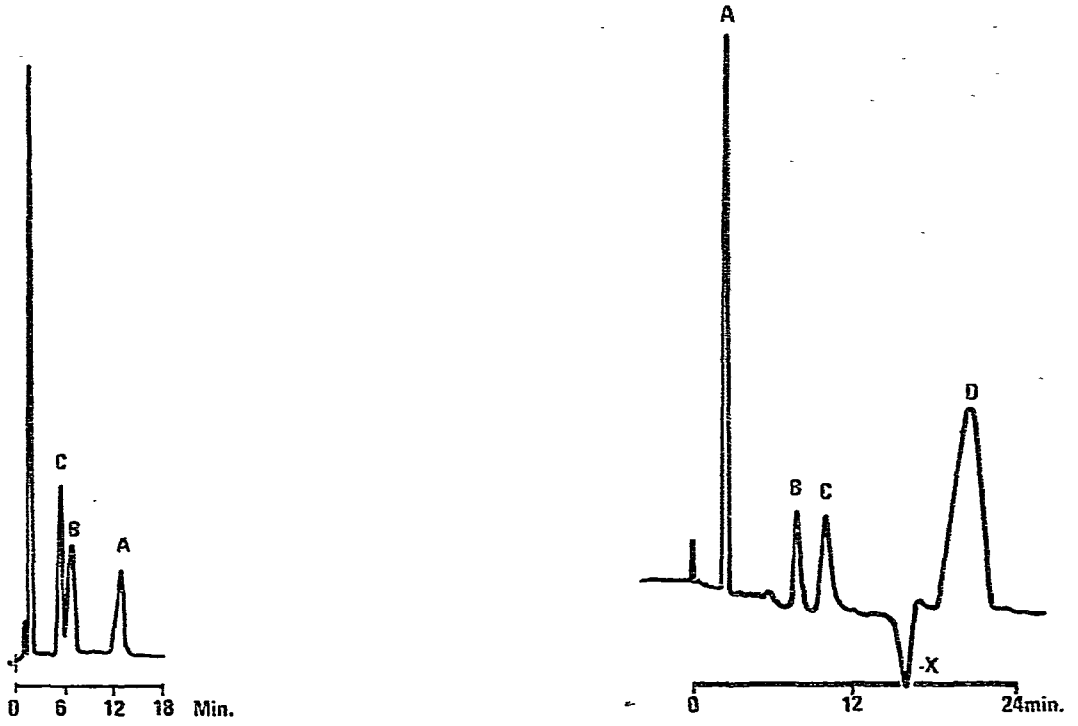

Fig. 4. Separation of carbohydrates in beverage. Column, 250 mm × 4.6 mm I.D., Partisil-10 PAC; mobile phase, acetonitrile–0.0025 *M* KH_2PO_4 (84:16), pH 5.0 with KOH; flow-rate, 110 ml/h; pressure, 375 p.s.i.; detector, refractive index, × 0.02. Amount injected: 7.0 µl. A = Sucrose; B = glucose; C = fructose.

Fig. 5. Separation of carbohydrates in maple sugar syrup. Column, 250 mm × 4.6 mm I.D., Partisil-10 PAC; mobile phase, acetonitrile–0.0025 *M* $NaC_2H_3O_2$, pH 5.0; flow-rate, 108 ml/h; pressure, 425 p.s.i.; detection, refractive index, 1.6×10^{-4}. Sample: pure maple sugar syrup; 25% of sample in water. A = Solvent front; B = fructose; C = glucose; D = sucrose; X = unidentified compound.

of pure maple sugar syrup. Component X is unidentified but is probably not a carbohydrate since it gives the only negative response on the refractive index detector that was observed throughout the study.

The food industry is supplementing traditional animal protein foods with vegetable protein in the form of soybean meal. The commonly occurring tetrasaccharide, stachyose, in soybean, however, causes gastric distress in humans and must be removed or reduced in concentration. The separation of a sample of soybean extract before any such removal is shown in Fig. 6.

Because people have to satisfy their "sweet tooth" yet want lower-calorie intakes, many artifical sweeteners have become available in recent years. Lactose is substituted for sucrose in such preparations. Fig. 7 shows the separation of lactose from saccharin in one commercially available artificial sweetener. A UV monitor placed in series with the refractive index monitor would allow for more sensitive detection of saccharin.

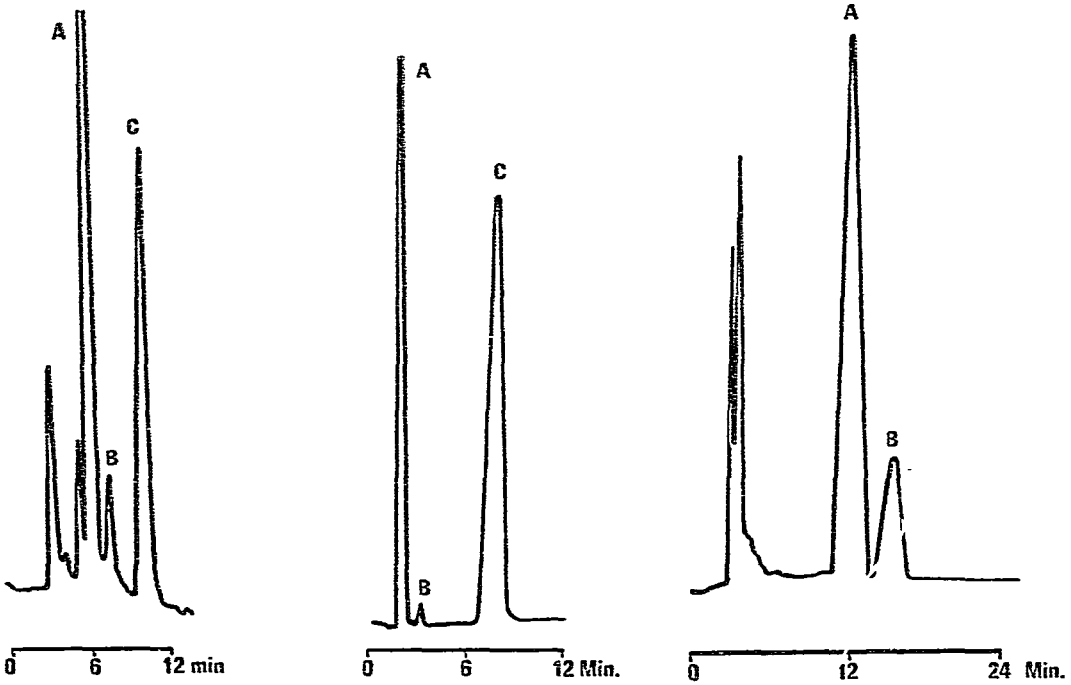

Fig. 6. Separation of carbohydrates in soybean extract. Column, 250 mm × 4.6 mm I.D., Partisil-10 PAC; mobile phase, acetonitrile–0.0025 *M* KH_2PO_4 (70:30), pH 5.0 with KOH; flow-rate, 70 ml/h; pressure, 300 p.s.i.; detector, refractive index, × 0.02. Sample: Soybean extract. A = Sucrose, 280 μg; B = raffinose, 84 μg; C = stachyose, 280 μg.

Fig. 7. Separation of carbohydrate in artificial sweetener. Column, 250 mm × 4.6 mm I.D., Partisil-10 PAC; mobile phase, acetonitrile–0.0025 *M* KH_2PO_4 (75:25), pH 5.0 with KOH; flow-rate, 92 ml/h; pressure, 465 p.s.i.; detector, refractive index, × 0.01–0.04. Sample: Sweet 'n Low®, 1 g/10 ml water. A = Solvent; B = saccharine, × 0.01; C = lactose, × 0.04.

Fig. 8. Separation of carbohydrates in intravenous high-calorie electrolyte solution. Column, 250 mm × 4.6 mm I.D., Partisil-10 PAC; mobile phase, acetonitrile–0.002 *M* KH_2PO_4, pH 5.0 with KOH; flow-rate, 90 ml/h; pressure, 250 p.s.i.; detection, refractive index, × 0.04; injected amount, 10 μl. A = Fructose; B = glucose.

Some hospitalized patients are given life-sustaining intravenous solutions; therefore, the quality control of such solutions is most important to pharmaceutical companies, the federal regulatory agencies, and the patients. Fig. 8 illustrates a separation, capable of precise quantitation and high reproducibility, of one such solution.

CONCLUSION

The separation of carbohydrates on Partisil-10 PAC with acid- or salt-modified acetonitrile/water combinations has been shown. This adjustment of pH or use of various anions in the mobile phase allows optimization of the separation but the mechanism of the observed changes with such modifiers is not clear, at present, since no distinct trends are noted. Complexation of the modifiers with the bonded phase or with the carbohydrates may be responsible, but further work will have to be per-

formed to furnish information upon which such a theory could be based. It was observed that the various classes of carbohydrates do fall within certain capacity factor ranges with any of the mobile phases used, as other researchers have found with similar chromatographic systems.

One of the advantages of performing separations in the partition mode is the improved column stability since interfering sample components seldom bond to the support. Filtration of samples is frequently the only clean-up step required in using the Partisil-10 PAC. The accompanying chromatograms show the applicability of this polar bonded phase to the separation of real samples containing carbohydrates using the refractive index detector. Only in the separation of the carbohydrate lactose in whole milk was an extra step (an extraction with carbon tetrachloride) used before sample injection.

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