Journal of Chromatography, 513 (1990) 227–235
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 440

Studies on the chromatographic behaviour of some uronic acids and neutral sugars on an amino-bonded phase column

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(First received August 8th, 1989; revised manuscript received December 29th, 1989)

ABSTRACT

The differences in the chromatographic behaviour of glucuronic and galacturonic acids and some common neutral sugars on an amino-bonded phase column (LiChrosorb NH_2) were studied. The mobile phase was either acetonitrile–water or acetonitrile–sodium dihydrogenphosphate buffer. The influences of the presence of an anion ($H_2PO_4^-$) in the mobile phase, the pH and the composition of eluent were investigated. The results contributed to the elucidation of the mechanism of the elution of uronic acids and the simultaneous determination of uronic acids and neutral sugars.

INTRODUCTION

The use of amino-bonded phase columns with a mixture of acetonitrile and water as eluent in high-performance liquid chromatographic (HPLC) separations of carbohydrates has been studied by numerous investigators[1–7]. However, most of the previous work dealt with the separation of neutral sugars and little attention has been paid to the separation of uronic acids with this method because uronic acids are not readily eluted from this type of column with aqueous acetonitrile systems. A few reports have been published on the separation of some organic acids on amino-bonded phase column[8–10]. An attempt to separate some uronic acids using a LiChrosorb 10 NH_2 column in a weak anion-exchange mode with sodium acetate buffers as eluent was not successful[10]. Further, the mechanism of the elution of uronic acids on this type of column is poorly known.

An alternative approach is to use ion-exchange columns for such separations. Most current procedures for separating acidic monosaccharides are carried out on anion- or cation-exchange resins[10–14]. In comparison with amino-bonded phase columns, ion-exchange columns offer different selectivities and elution sequences, but have to be run at high temperatures and are either prone to rapid degeneration[10,15] or have little facility for retention time adjustment[15,16]. Comparative studies[15] and critical reviews[16] concerning liquid chromatographic carbohydrate separations on the above columns have been published.

0021-9673/90/$03.50

Based on these observations, we were interested in extending the potential of amino-bonded phase columns to the simultaneous analysis of neutral sugars and uronic acids. It is of value to analyse directly the uronic acids and neutral sugar residues in order to identify the polysaccharides as it is the first step in the structural analysis of polysaccharides. In this paper, we report an investigation of the chromatographic behaviour of uronic acids and neutral sugars on an amino-bonded phase column and discuss the results from the viewpoint of structural features and elution mechanism. The possibility of the simultaneous determination of uronic acids and neutral sugars is considered.

EXPERIMENTAL

Materials

Standard saccharide samples were obtained from Sigma and Aldrich. HPLC-grade acetonitrile (Fluka) and analytical-reagent grade sodium dihydrogenphosphate (NaH_2PO_4) were used.

Preparation of hydrolysate of acidic polysaccharides. An acidic polysaccharide, PIA-I, prepared in our laboratory, was hydrolysed with 2 *M* trifluoroacetic acid (TFA) at 120°C for 1 h in a sealed tube. After the TFA had been removed by evaporation, the hydrolysate was dissolved in water, filtered to remove any particulate matter and then subjected to HPLC analysis.

Apparatus

The equipment consisted of a programmable HPLC pump (Waters Model 590) a differential refractometer (Shodex Model SE-51) and a high-pressure sampling valve (Bio-Rad Model 7125). The detector output was recorded with a flat-bed recorder (LKB Model 2210) and an integrator (CDMC-2, China).

Chromatography was carried out with a 200 × 5 mm I.D. stainless-steel column packed with 5-μm LiChrosorb NH_2 (E. Merck) and a 5 × 5 mm I.D. guard column packed with the same material.

Chromatographic conditions

The mobile phase was a mixture of acetonitrile and phosphate buffer or water. All solvents were filtered and degassed ultrasonically before use. The column was equilibrated overnight with the respective eluent at a flow-rate of 0.5 ml/min and then the flow-rate was maintained at 1 ml/min during chromatography. A 20-μl volume of a sample solution containing 0.5–2% (w/w) of each saccharide was applied to the column. All separations were done at room temperature and no attempt was made to control the temperature of the mobile phase and the column.

RESULTS AND DISCUSSION

A typical separation of some common mono- and disaccharides using acetonitrile–water (80:20, v/v) (pH 8.4) as eluent is shown in Fig. 1. The elution sequence and resolution are similar to those reported using the same column[1,3–5]. With this LiChrosorb NH_2 column and acetonitrile–water as eluent, however, uronic acids such as glucuronic acid (GlcA) and galacturonic acid (GalA) could not be eluted as

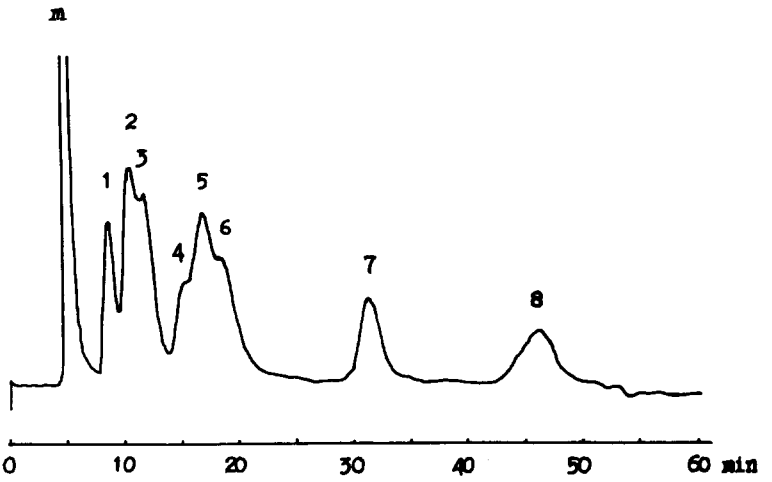


Fig. 1. Separation of a neutral mono- and disaccharide mixture on LiChrosorb NH_2 column. Flow-rate, 1.0 ml/min. Eluent, acetonitrile–water (80:20, v/v) (pH 8.4). Peaks: 1 = rhamnose; 2 = xylose + fucose; 3 = arabinose; 4 = mannose; 5 = glucose; 6 = galactose; 7 = sucrose; 8 = lactose; m = mobile phase.

measurable peaks within a reasonable time. For the separation of these acids, a phosphate buffer–acetonitrile solvent was tested[6]. Fig. 2 shows the chromatogram obtained on the same column with acetonitrile–0.015 *M* NaH_2PO_4 buffer (80:20, v/v) (pH 7.3) as eluent for the separation of a standard mixture of uronic acids and neutral sugars. GlcA was eluted as a comparatively broad peak but GalA was still not eluted.

The marked difference in the elution behaviour of the uronic acids with the two solvent systems led us to investigate the effects of the composition and pH of the buffered mobile phase on the separation of the acids.

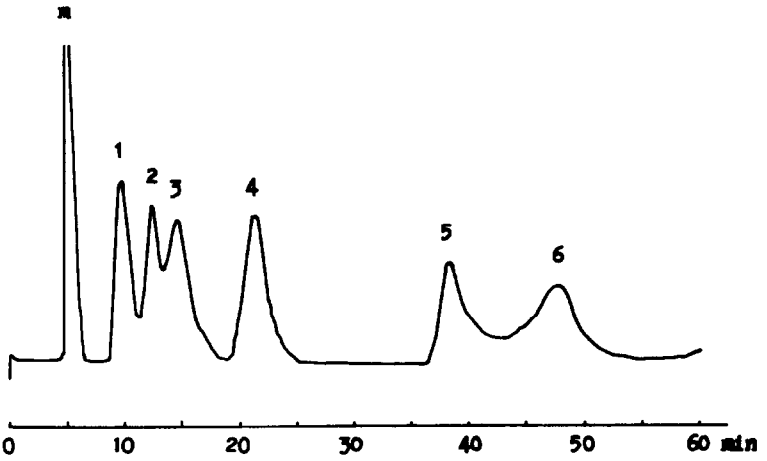


Fig. 2. Separation of (1) rhamnose, (2) xylose, (3) arabinose, (4) glucose, (5) sucrose, (6) glucuronic acid and galacturonic acid on the same column. Flow-rate, 1.0 ml/min. Eluent, acetonitrile–0.015 *M* NaH_2PO_4 (80:20, v/v) (pH 7.3).

At fixed acetonitrile to buffer ratio of 75:25 (v/v), the pH of the mobile phase was adjusted to 7.2 and 6.9 with 0.015 and 0.018 *M* NaH_2PO_4, respectively. Using these eluents, the same test mixture as in Fig. 2 was chromatographed and the results are shown in Fig. 3. On comparing the elution profiles of GlcA and GalA in Fig. 3B with that in Fig. 3A, the improvement in peak width and peak tailing obtained with the eluent of lower pH is obvious, especially for GalA, although a negative peak appeared at the retention time of GalA and split the relatively broad peak of GalA into two. This negative peak has also been reported by Hendrix *et al.*[17] using a similar system with amine-modified silica as the stationary phase, and was found to be a function of the eluent pH. The acidic eluents resulted in a strong negative peak following trisaccharide elution.

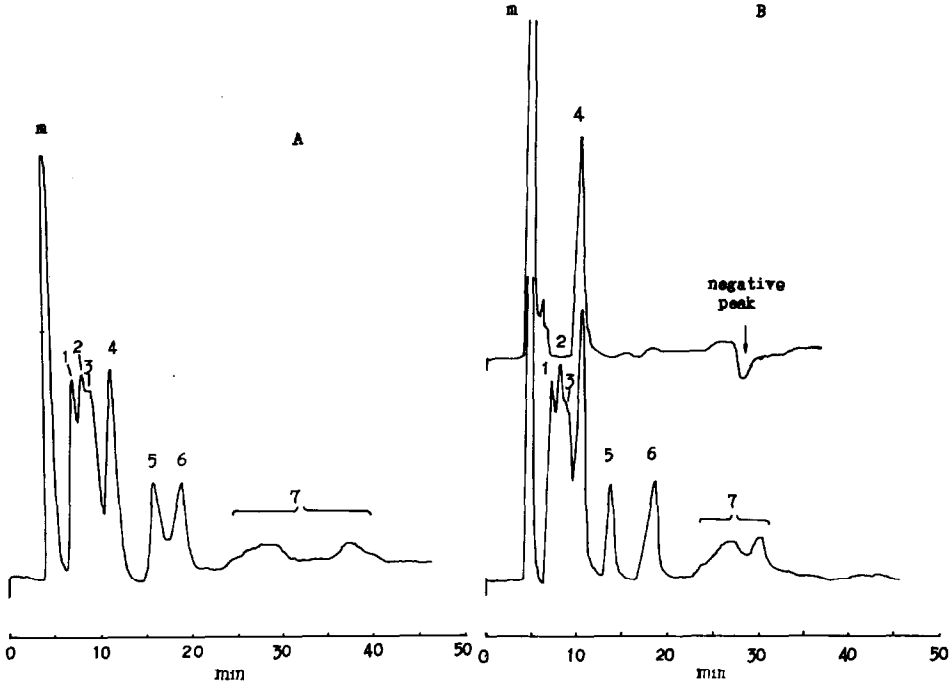


Fig. 3. Effect of increasing the acidity of eluent on solute elution. Flow-rate, 1.0 ml/min. Eluents: A, acetonitrile–0.015 *M* NaH_2PO_4 (75:25, v/v) (pH 7.2); B, acetonitrile–0.018 *M* NaH_2PO_4 (75:25, v/v) (pH 6.9). Note that a negative peak appears at the GalA elution position. Peaks: 1 = rhamnose; 2 = xylose; 3 = arabinose; 4 = glucose; 5 = sucrose; 6 = glucuronic acid; 7 = galacturonic acid.

In our system, a solvent with a higher water content was employed to elminate this undesirable negative peak. Fig. 4 illustrates the results for the test mixture and GalA alone with acetonitrile–0.015 *M* NaH_2PO_4 (70:30, v/v) (pH 7.0) as eluent. GlcA and GalA were eluted as symmetrical peaks and a baseline separation was obtained.

In Table I, the retention times and capacity factors (k') of the carbohydrates determined with various solvents are presented. The retention of the carbohydrates on the amino-bonded phase is strongly dependent on structural features. It has been proposed that the number and geometry of hydroxyl groups on the neutral sugars are

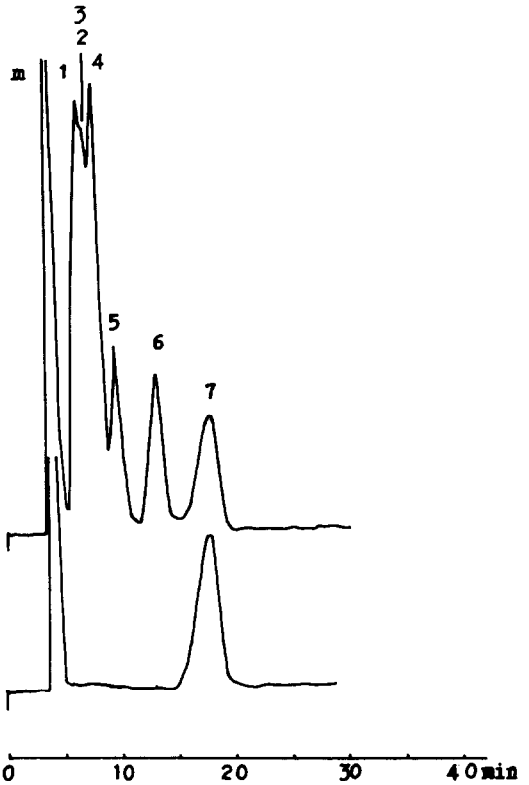
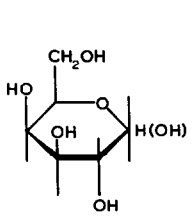
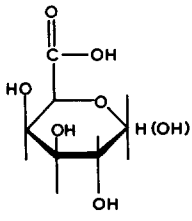


Fig. 4. Chromatogram obtained with a higher water content in the buffered solvent. Flow-rate, 1.0 ml/min. Eluent, acetonitrile–0.015 *M* NaH_2PO_4 (70:30, v/v) (pH 7.0). Peaks as in Fig. 3.

important factors for separation[18]. In general, more hydroxyl groups would result in more hydrogen bonds with the stationary phase, therefore increasing the retention. The uronic acids are distinguished from neutral monosaccharides in the replacement of the 6-hydroxyl group with a carboxylate group:



D-Galactose



D-Galacturonic acid

Our data indicate that a uronic acid either cannot be eluted from the column or is eluted much later than the corresponding neutral sugar. This different behaviour cannot be explained by the difference in the number of hydrogen bonds.

TABLE I

RETENTION DATA FOR NEUTRAL SUGARS AND URONIC ACIDS ON AN AMINO-BONDED PHASE COLUMN WITH DIFFERENT MOBILE PHASE SYSTEMS

Sample	*No. of hydroxyl groups*	*System I: CH_3CN–water (80:20, v/v) (pH 8.4)*		*System II: CH_3CN–0.015 M NaH_2PO_4 (80:20, v/v) (pH 7.3)*		*System III: CH_3CN–0.015 M NaH_2PO_4 (75:25, v/v) (pH 7.2)*		*System IV: CH_3CN–0.018 M NaH_2PO_4 (75:25, v/v) (pH 6.9)*		*System V: CH_3CN–0.015 M NaH_2PO_4 (70:30, v/v) (pH 7.0)*	
		t_r (min)	*k'*	*t_r (min)*	*k'*	*t_r (min)*	*k'*	*t_r (min)*	*k'*	*t_r (min)*	*k'*
Rhamnose	4	8.84	0.740	9.73	0.966	7.38	0.689	6.90	0.612	6.44	0.656
Xylose	4	10.50	1.067	12.42	1.509	8.48	0.941	7.76	0.813	6.68	0.717
Fucose	4	10.70	1.106	12.71	1.568	8.62	0.973	7.86	0.836	6.71	0.725
Arabinose	4	12.10	1.382	14.59	1.947	9.27	1.121	8.63	1.016	6.82	0.753
Mannose	5	15.79	2.108	18.76	2.790	10.75	1.460	9.49	1.217	7.57	0.946
Glucose	5	17.10	2.366	21.56	3.356	11.54	1.641	10.09	1.357	7.99	1.054
Galactose	5	18.30	2.602	22.85	3.616	12.03	1.753	10.39	1.428	8.21	1.111
Sucrose	8	30.67	5.037	38.09	6.695	16.12	2.689	13.61	2.180	9.73	1.501
Lactose	8	46.31	8.116	53.33	9.774	20.12	3.604	17.81	3.161	11.35	1.918
GlcA	4	[a]	–	47.12	8.519	19.26	3.407	18.49	3.320	13.14	2.378
GalA	4	[a]	–	[a]	–	[b]	–	[b]	–	17.85	3.589
t_0		5.08		4.95		4.37		4.28		3.89	

[a] Sample cannot be eluted from the column.
[b] Peaks too broad to measure.

It is also interesting that GlcA was eluted before or after the disaccharide lactose with the different mobile phases employed. It has been observed previously that when neutral mono- or disaccharides were separated on an amino-bonded phase column, although the capacity factors were a function of the eluent pH, composition and the column used, the elution order was not significantly effected by them[6,7,17,19]. Our results on neutral sugars are also in agreement with these reports. This can probably be attributed to the dominant force in the separation of these neutral sugars on the amino-bonded phase column being the same as forces such as hydrogen bonding force[1,6,18]. If the retention of uronic acids on the columns were also caused by this force, GlcA would follow the same rule and the above factors would not change its elution sequence. When the eluent pH and composition were varied, however, the experimental results did not support the above assumption. The influence of the pH and composition of the eluent on the retention order of uronic acids and neutral sugars is demonstrated in Fig. 5.

These results may lead to a better understanding of the elution mechanism of the acids. It seems more likely that the retention of uronic acids is mainly derived from other kinds of molecular forces. This may also explain why the uronic acid cannot be eluted from LiChrosorb NH_2 with an acetonitrile–water system. Some similar organic acids on an amino-bonded phase column were presumed to form salts with the amino groups in the normal-phase mode[8].

From literature data and our studies, it appeared that the retention of carbohydrates decreased with decreasing eluent pH. However, we also noticed that

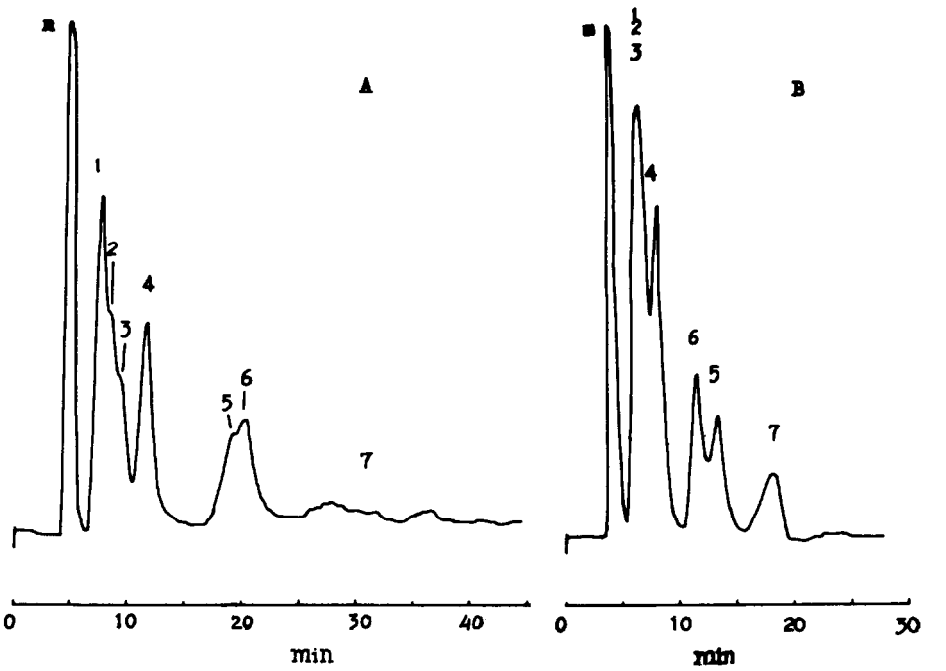


Fig. 5. Influence of eluent modification on the elution order of uronic acids and neutral sugars. Flow-rate, 1.0 ml/min. Eluents: A, acetonitrile–0.015 *M* NaH_2PO_4 (75:25, v/v) (pH 7.2); B, acetonitrile–0.015 *M* NaH_2PO_4 (70:30, v/v) (pH 7.0). Peaks: 1 = rhamnose; 2 = xylose; 3 = arabinose; 4 = glucose; 5 = glucuronic acid; 6 = lactose; 7 = galacturonic acid.

increased retention times for neutral sugars were obtained in system II, the eluent pH being changed from 8.4 in system I to 7.3 with phosphate buffer. It was considered possible that the role of the anion ($H_2PO_4^-$) in the mobile phase is not only to adjust the eluent pH, but also to form a complex with the bonded phase. The complex is probably similar to the amine phosphate salt [$RNH^-O^+PO(OH)_2$] obtained on a phosphoric acid-modified amino-bonded phase column[20]. Preliminary investigations indicated this modified amino-bonded phase column did not change the elution order of neutral sugars but had a significant influence on the elution of GlcA and GalA. Further work is in progress.

From the above it is evident that the addition of NaH_2PO_4 to buffer the eluent is essential for elution of the acids from the column and a higher water content of the eluent can eliminate the negative peak found with acidic eluents and have a promoting effect on the elution of the uronic acids.

Accordingly, under the appropriate mobile phase conditions, the method described makes it possible to analyse directly the hydrolysate of polysaccharides which contain uronic acid units. Fig. 6 demonstrates the result for a real sample on the amino-bonded phase column. Filtration of the sample is the only pretreatment and no derivatization is required. The column used in our system appeared to be stable.

In conclusion, owing to the different chromatographic behaviour between uronic acids and neutral sugars on an amino-bonded phase column, the use of

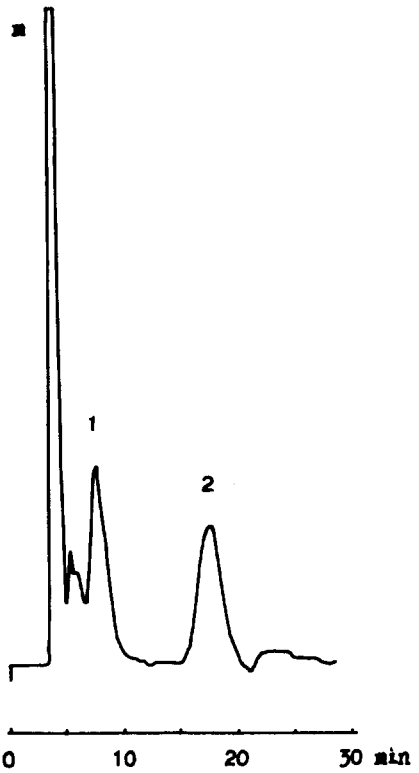


Fig. 6. Separation and determination of (1) galactose and (2) galacturonic acid in the hydrolysate of the acidic polysaccharides PIA-I. Flow-rate, 1.0 ml/min. Eluent: acetonitrile–0.015 *M* NaH_2PO_4 (70:30, v/v) (pH 7.0).

a buffered mobile phase and control of the eluent pH and composition are necessary for the simultaneous and direct analysis of these carbohydrate mixtures. This HPLC method has proved helpful in the study of the structural analysis of acidic polysaccharides such as plant gums and mucilages.

ACKNOWLEDGEMENT

The authors are grateful to the Chinese Foundation of Natural Sciences for financial support of this work.

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