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ANALYSIS OF URONIC AND ALDONIC ACIDS, THEIR LACTONES, AND RELATED COMPOUNDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON CATION-EXCHANGE RESINS

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SUMMARY

The use of high-performance gel-permeation chromatography on cation-exchange resins for the direct analysis of 21 examples of the title compounds is described. The method employs a commercially available column (HPX-87-H^+), a simple isocratic solvent system (0.009 *N* sulfuric acid), and sensitive UV detection at 220 nm. Compounds are rapidly (less than 15 min) separated by a combination of ion- and size-exclusion mechanisms, leading to the following general elution sequence: aldonic and uronic acids, then ascorbic acids, followed by neutral lactones, and finally N-acetylated amino sugars. The method is a useful, high-resolution alternative to the traditional gas chromatographic and anion-exchange chromatographic methods for the analysis of these compounds.

INTRODUCTION

Uronic and aldonic acids are naturally occurring compounds that have a broad range of important biological and chemical functions. The uronic acids, for instance, are major components of ion-regulatory and structural polysaccharides in plants and algae, and are also found in the glycosaminoglycans of animal tissue. The involvement of both uronic[1] and aldonic[2] acids in metabolism has been established, and certain of their lactones[3] are now known to be selective inhibitors of glycosidases. Other aldonic acids and lactones are sequestering agents[4] for metal ions and have important industrial and drug applications.

Because of the many important roles that uronic and aldonic acids and their lactones play, it is important to have accurate and rapid methods for their analysis. The presently available methods that involve colorimetry[5], gas–liquid chromatography[6–9], or anion-exchange chromatography[10–13] each possess unique limitations: non-specificity, the need for the preparation of volatile derivatives, and long (1–6 h) analysis times, respectively. High-performance anion-exchange chromatography[14–16] provides rapid, high resolution separations of uronic acids, but the commercially available packing materials are unstable and prone to rapid degeneration.

The use of cation-exchange resins as a stationary phase for the chromatographic analysis of various water-soluble acidic and neutral compounds has recently gained popularity[17]. Separations of this type, which do not rely on ion-exchange phenomena, *per se,* were pioneered by Wheaton and Bauman[18,19], who demonstrated that mixtures of electrolytes and non-electrolytes could be easily separated on cation exchangers when water was used as an eluent. In this ion-exclusion process, ionic solutes pass through the interstitial volume of the resin bed, but are prevented from entering the pore volume of the packing gel because of Donnan membrane effects. Ionic solutes therefore appear in the column effluent at a point equal to the interstitial, or exclusion volume. Non-ionized solutes and weak electrolytes, which are able to penetrate the internal volume of the packing gel, will elute at a later volume that is determined by the extent of the solutes permeation into, or interactions with the gel matrix. The process can be described by the following equation[19]:

$$V_{max} = V_0 + K_d V_i \quad (1)$$

which defines the retention volume of a solute (V_{max}) in terms of the column interstitial volume (V_0) and the fraction (K_d) of the internal pore volume (V_i) of the gel that the solute can occupy during elution. For a true exclusion process, all solutes would be expected to elute between the column volumes of V_0 ($K_d = 0$, complete exclusion) and $V_0 + V_i$ ($K_d = 1$, no exclusion). For two solutes to be separated, therefore, they must possess sufficiently different K_d values. Wheaton and Bauman[19] and others[20–22] demonstrated that the K_d value of any given solute is determined by its pK_a, molecular weight, London or van der Waals forces between solute and resin matrix, and polar interactions between solute and the fixed resin ionic groups. Because of the relatively wide variety of sorption and exclusion effects demonstrated in those[19–22] systems, simple mixtures of ionic and non-ionic solutes could be separated. More recently, two additional refinements have improved the efficiency and the selectivity of the exclusion methods. First, the use of high-performance columns, efficiently packed with small (5–10 μm), spherical resins[23] allows more efficient mass transfer of solute between stationary and mobile phases, resulting in larger numbers of theoretical plates per column. A second advance is the use of dilute mineral acid in the mobile phase[17,24–26] to increase the selectivity of the column towards ionized solutes. In this modification, the acidic mobile phase suppresses the ionization of weak acids, allowing them to penetrate the Donnan boundary and partition into the resin pore volume. The result is longer retention times, greater solute–matrix interactions, and better overall column selectivity.

The use of these gel permeation methods for the separation of ascorbic acid and related compounds[27], selected uronic acids and lactones[28], and gluconic acid[29] have recently been described. The results of those limited studies suggest that high-performance liquid chromatography (HPLC) on cation-exchange resins may be a valuable general method for analysis of many carbohydrate-derived acids and lactones. We have therefore conducted a more detailed study on the use of this method for the individual analysis of 21 different types of uronic acids, aldonic acids, their lactones, and related compounds such as ascorbic acids and keto-aldonic acids. Because of their frequent natural occurrence with the uronates, the N-acetylated hexosamines also were studied. The use of this chromatographic system for the analysis

of these compounds in model systems, reaction mixtures, and biological samples is demonstrated.

EXPERIMENTAL*

Materials

D-Glucuronic acid, D-galacturonic acid, D-gluconic acid (potassium salt), D-galactonic acid (calcium salt), 2-ketogluconic acid (calcium salt), 5-ketogluconic acid (potassium salt), D-glucurono-6,3-lactone, D-mannurono-6,3-lactone, L-mannono-1,4-lactone, D-ribono-1,4-lactone, D-galactono-1,4-lactone, 2-acetamido-2-deoxy-D-glucose, and 2-acetamido-2-deoxy-D-galactose were purchased from Sigma. Other experimental compounds and their sources were: D-glucono-1,5-lactone, U.S. Biochemicals; D-glycero-D-guloheptono-1,4-lactone, Pfanstiehl; ascorbic acid, Fisher; erythorbic acid ("isoascorbic acid"), Aldrich. D-Mannuronic acid, D-glycero-D-guloheptonic acid, L-mannonic acid, and D-ribonic acid were all prepared by the saponification of their respective lactones.

Liquid chromatography

All chromatography was performed with a DuPont Model 8800 pump and heated column compartment, a Rheodyne fixed loop (20 μl) injector, Gilson Holochrome UV detector, and Hewlett-Packard 3390 integrator. The column was a BioRad HPX-87-H^+ model (30 cm × 7.8 mm I.D.) packed with 9 μm, spherical, sulfonated polystyrene–divinylbenzene beads with 8% cross-linking, eluted with filtered (0.2-μm nylon 66 filter) 0.009 *N* sulfuric acid. A BioRad precolumn containing strong cation-exchange resin was used throughout the study.

Standards were prepared daily by dissolving pure sugar acids, their salts, or lactones in HPLC-grade water. Solutions were filtered through 0.2-μm nylon 66 filters prior to injection.

The UV detectability of each model compound was determined in the following manner. Known concentrations of pure lactone or free acid (over a range of 0.25 to 10 mg/ml) were chromatographed in duplicate, and the resulting peak heights were plotted *versus* concentration. These two parameters were fitted to a line by linear regression analysis. The slope of the line was used as the specific detector response of each compound and the correlation coefficient was used to define the linearity of the detector response over that concentration range.

Hydrolysis of polysaccharides

Acid hydrolysis. Polygalacturonic acid (200 mg; Sigma), dispersed in 1-ml of ice-cold 80% sulfuric acid, was allowed to sit at 25°C for 18 h. The sample was then diluted to 13 ml, sealed in a vial, and placed in a boiling water bath for 5 h. The resulting dark solution was neutralized with solid calcium carbonate, filtered (0.2 μm), and injected into the chromatograph.

Enzyme hydrolysis. Polygalacturonic acid (5 g), dissolved in 60 ml of water, was titrated to pH 4 with *N* sodium hydroxide (13 ml). Sigma pectinase (1250 units)

* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

was added, the solution was stirred, and toluene (5 ml) was added to prevent microbial growth. After incubation for 40 h at 25°C, the mixture was evaporated at 35°C under reduced pressure. The thick syrup was then reconstituted to 50 ml with water, passed through a column of IR-120-H^+ (50 ml), and finally, filtered (0.2 μm) prior to HPLC analysis.

Analysis of bacterial metabolites

Bacterial colonies, selected from solid growth media previously inoculated with plant root washings, were grown in a previously described media[30]. At selected growth intervals, aliquots of growth media (2 ml) were treated with IR-120-H^+ resin (1.2 g). After removal of the resin, this solution was mixed with acetonitrile in the ratio 1:5. This solution was shaken for 1 min, passed through a filter (0.2 μm), and analyzed directly by HPLC.

Identification of 2-ketogluconic acid

The 2-ketogluconic acid produced in growth media (see Results and Discussion, Fig. 8) was positively identified by ^{13}C-NMR spectroscopy, after it was isolated by preparative anion-exchange chromatography. Thus, 17 ml of growth media were first treated with IR-120-H^+ and acetonitrile in the same ratios as described above. This solution was then loaded onto a column containing 10 ml of Duolite A-561 (free base form). Neutral compounds were eluted with water (35 ml), and 50% formic acid (10 ml) was used to elute the acidic fraction, which was subsequently lyophilized. The ^{13}C-NMR spectrum of this isolated fraction showed the presence only of chemical shifts identical with that of authentic 2-ketogluconic acid.

RESULTS AND DISCUSSION

Retention behavior of standard compounds

The retention of 21 different sugar-derived acids, lactones, and N-acetylated compounds was examined on a commercial column packed with strong cation-exchange resin (H^+ form) using 0.009 *N* sulfuric acid as the mobile phase. Figs. 1–3 illustrate the relative retention times of these compounds at a variety of temperatures. In this system, temperature plays an important role in the apparent column selectivity for each compound, and can be effectively used to optimize a particular separation. These temperature effects are not only chromatographically useful, but also informative, since they provide insight into the chemical stability of, and dynamic equilibrium between various acid/lactone pairs.

It is well known that aldonic and uronic acids may be converted into, or equilibrated with, their various lactone forms, and that the equilibrium concentration of each species is determined by pH, temperature, and solvent effects. Being able to determine accurately the amount of each form present under various conditions would be helpful for determining which species is involved in certain chemical or biological events, and also for determining the best synthetic route to a desired form. From Figs. 1–3, it is apparent that conditions exist for the simultaneous separation and independent quantification of each member in most of the acid/lactone pairs.

The configurational stability of various acids and lactones may also be inferred from Figs. 1–3, and these data are consistent with previously reported information[31].

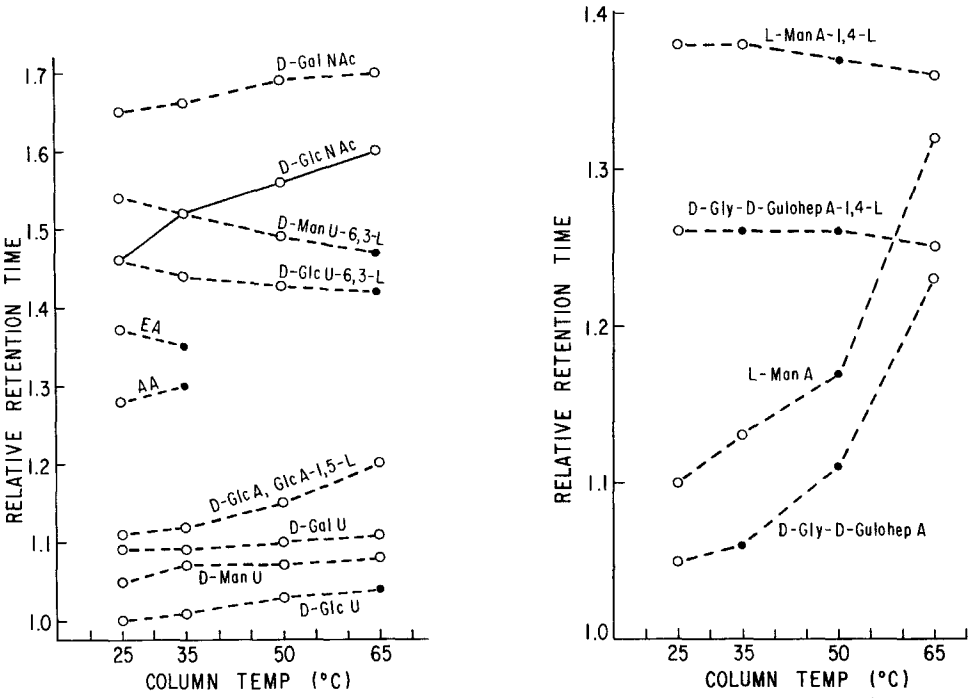

Fig. 1. Effect of temperature on relative retention times of various compounds. HPX-87-H$^+$ column, eluted with 0.009 *N* sulfuric acid at 0.6 ml/min. UV detection at 220 nm. All retention times are relative to that of D-glucuronic acid at 25°C (7.8 min). Open circles represent single, symmetrical peaks; closed circles represent broad or complex peaks. Abbreviations: D-GalNAc = 2-acetamido-2-deoxy-D-galactose; D-GlcNAc = 2-acetamido-2-deoxy-D-glucose; D-ManU-6,3-L = D-mannurono-6,3-lactone; D-GlcU-6,3-L = D-glucurono-6,3-lactone; EA = erythorbic acid; AA = ascorbic acid; D-GlcA = D-gluconic acid; D-GlcA-1,5-L = D-glucono-1,5-lactone; D-GalU = D-galacturonic acid; D-ManU = D-mannuronic acid; D-GlcU = D-glucuronic acid.

Fig. 2. Effect of temperature on relative retention times of various compounds. Abbreviations: L-ManA-1,4-L = L-mannono-1,4-lactone; D-Gly-D-GulohepA-1,4-L = D-glycero-D-guloheptono-1,4-lactone; L-ManA = L-mannonic acid; D-Gly-D-GulohepA = D-glycero-D-guloheptonic acid. Other conditions as in Fig. 1.

Consider first, the aldonic acids and lactones. It is well known that at high temperatures and low pH, the aldonic acids are converted partially into 1,5- and 1,4-lactones. This fact is reflected in the gradual increase in retention times of all aldonic acids as temperature increases. At the highest temperature studied, 65°C, D-galactonic, D-ribonic, D-glycero-D-guloheptonic, and L-mannonic acids all possess much longer (lactone-like) retention times, which are either identical with, or very similar to those of their respective aldono-1,4-lactones. In fact, re-chromatography (at 25°C) of the D-galactonic acid peak that was collected from a 65°C chromatographic run, gives a peak with an identical retention time to that of D-galactono-1,4-lactone. This suggests that many aldonic acids and 1,4-lactones may be determined separately at low temperature, and collectively at higher temperature.

Aldono-1,4-lactones are generally more stable than 1,5-lactones[31]. The 1,4-lactones studied here, especially D-glycero-D-guloheptono-1,4-lactone (Fig. 2), L-

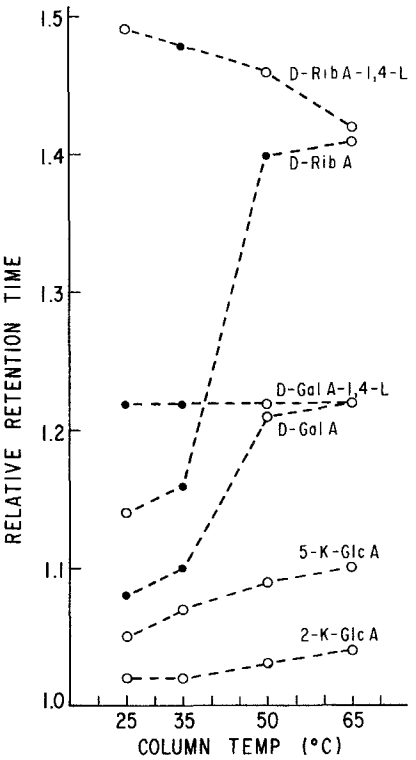

Fig. 3. Effect of temperature on relative retention times of various compounds. Abbreviations: D-RibA-1,4-L = D-ribono-1,4-lactone; D-RibA = D-ribonic acid; D-GalA-1,4-L = D-galactono-1,4-lactone; D-GalA = D-galactonic acid; 5-K-GlcA = 5-ketogluconic acid; 2-K-GlcA = 2-ketogluconic acid. Other conditions as in Fig. 1.

mannono-1,4-lactone (Fig. 2), and D-galactono-1,4-lactone (Fig. 3) all gave consistent retention times, and no indication of hydrolysis as the temperature of analysis increased. On the other hand, D-glucono-1,5-lactone, the only 1,5-lactone studied here, is known to be very unstable[32] and subject to hydrolysis in aqueous solution. This compound chromatographed in a manner consistent with these facts; it coeluted with D-gluconic acid, under all the conditions examined (Fig. 1).

The uronic acids and lactones also were well separated by this system (Fig. 1). The uronic acids are more stable towards lactonization under these conditions than are the aldonic acids, and they give sharp, symmetrical peaks at all but the highest temperatures studied. At 65°C D-glucuronic acid (and to a lesser extent, D-mannuronic acid) gave broad peaks that indicated partial lactonization. The formation of a 6,3-lactone from D-galacturonic acid is reported to be stereochemically unfavorable[33] and this is confirmed by the single, sharp peak given by this compound, even at 65°C.

The N-acetylated sugars (Fig. 1) gave sharp, symmetrical peaks, with longer retention times than those of most acids and lactones. These retention times increased with temperature. Ascorbic and isoascorbic acid (Fig. 1) are only moderately well separated on this system, and the tendency for these compounds to decompose is

great, even at the lower temperatures. Analysis of these compounds by this method is therefore not recommended. The 2- and 5-"keto"-aldonic acids (Fig. 3) however, gave sharp, symmetrical peaks at every temperature studied.

It is noteworthy that the molecules investigated in this study appear to be separated by a combination of size- and ion-exclusion mechanisms. Using the method of Tanaka *et al.*[34], it was determined that the exclusion volume (V_0) of the column (3.74 ml, under these experimental conditions) is 32% of the total column liquid volume (V_t = 11.60 ml). The elution range of the compounds studied here varied from 4.75 ml (41% of V_t) to 13.43 ml (70% of V_t). The fact that all compounds are eluted well within one column volume lends support to exclusion as being a major mechanism in these separation processes. In order to partially supress ion-exclusion effects[24–26,28] and to increase the retention of the sugar acids in this study, the acidity of the mobile phase was adjusted to pH 2. Still, under these conditions, ion-exclusion plays a significant role in the separation process, as suggested by the general elution sequence of sugar acids, then weakly acidic lactones and finally, neutral molecules. In addition, the elution sequence of the three isomeric hexuronic acids, D-glucuronic acid (pK_a 3.20), D-mannuronic acid (pK_a 3.38), and D-galacturonic acid (pK_a 3.42), is consistent with this mode. The importance of size-exclusion effects in this system is also evident from the fact that even the non-ion-excluded neutral lactones are eluted well with one total column volume. In addition, the elution order of the lactones are approximately in order of their decreasing molecular weight, as would be predicted for size-exclusion chromatography. Moreover, size exclusion[35,36] has been suggested as a major factor in the separation of neutral saccharides on cation-exchange resins. The fact that isomeric lactones or isomeric neutral N-acetylated amino sugars are well separated in this system indicates that additional non-exclusion[19,21,22] separation mechanisms may also be involved.

Quantitative aspects

Linear regression analyses were performed to determine the relationship between UV detector response and the concentrations of selected acids and lactones that were injected. Under the conditions employed, all the compounds tested (Table I) were readily detectable and gave linear peak height responses for concentrations from 0.25 to 10.0 mg/ml. Microdetectability was determined for one compound, D-glucuronic acid, at 35°C. This compound was easily detected at a concentration of 25 μg/ml. Relative detector response values (Table I) varied considerably from one compound class to another. Lactones generally gave lower detector response values than acids. Response values for a given compound also varied considerably from one analysis temperature to another. For quantitative analysis, therefore, it is necessary to run analytical standards under precisely the same conditions as the sample being analyzed.

Most of the compounds in Table I were analyzed under conditions that were shown (Figs. 1–3) to produce sharp, symmetrical peaks (see legend, Fig. 1). Analysis at these temperatures leads to the enhanced detectability and resolution of closely eluting peaks. Certain situations exist, however, where compounds must be chromatographed under conditions that lead to broad, slightly asymmetrical peaks. An example of this is in the simultaneous separation of D-galactonic acid and D-galactono-1,4-lactone, which can only be accomplished at 25–35°C (Fig. 3). At these tem-

TABLE I

CHROMATOGRAPHIC PROPERTIES OF SELECTED COMPOUNDS

HPX-87-H^+ column, 0.009 *N* sulfuric acid mobile phase, 0.6 ml/min flow-rate. UV detection at 220 nm.

Compound	*Temp. (°C)*	*Relative* retention time*	*Relative detector response***	*Linearity****
D-Glucuronic acid	35	1.01	1.00	0.998
2-Ketogluconic acid	35	1.01	4.13	0.992
5-Ketogluconic acid	35	1.06	0.85	0.999
D-Galacturonic acid	35	1.09	0.66	0.998
D-Gluconic acid	35	1.11	1.24	0.996
D-Glucono-1,5-lactone	35	1.11	1.31	0.990
D-Galactonic acid	25	1.06	0.93	0.995
	35	1.09	0.60	0.992
	65	1.22	0.97	0.999
D-Galactono-1,4-lactone	25	1.22	1.03	0.999
	50	1.23	1.10	0.998
	65	1.22	1.03	0.999
D-Glycero-D-guloheptono-1,4-lactone	65	1.25	0.63	0.999
L-Mannono-1,4-lactone	35	1.38	0.62	0.999
	65	1.36	0.68	0.999
D-Ribono-1,4-lactone	65	1.42	0.72	0.995
D-Glucurono-6,3-lactone	35	1.44	0.58	0.999
D-Mannurono-6,3-lactone	35	1.52	0.49	0.999
D-Glucose	35	1.17	0.009	0.954
D-Galactose	35	1.24	0.007	0.950

* Retention time, relative to that of D-glucuronic acid at 25°C (7.8 min).

** Detector response of each compound, relative to D-glucuronic acid at 35°C. See Experimental section.

*** Correlation coefficient from linear regression analysis. See Experimental section.

peratures each compound gives broad chromatographic profiles. Linear regression analysis revealed, however (Table I), that even under these conditions calibration curves were linear and the analysis can be performed accurately.

It is noteworthy, that certain samples, such as cell wall polysaccharide digests, contain neutral sugars in addition to uronic acids. The neutral sugars tested here, however, do not elute in the same retention time range (Table I) as most uronic and aldonic acids. The detector response for these compounds is also quite low (Table I) relative to the sugar acids. Hence, the presence of neutral sugars will have no discernable effect on quantitation of the compounds studied here except in the most extreme cases.

Model separations

Fig. 4 demonstrates the ability of this system to simultaneously analyze closely related uronic acids and their lactones. With this system, mixtures of pure D-glucuronic (1) and D-mannuronic (2) acids can be separated under conditions (35°C) that do not promote their lactonization (notice the lack of lactone peaks in Fig. 4a). Likewise, the same conditions separate D-glucurono-6,3-lactone (3) and D-mannurono-6,3-lactone (4) without promoting hydrolysis to their respective acids. The

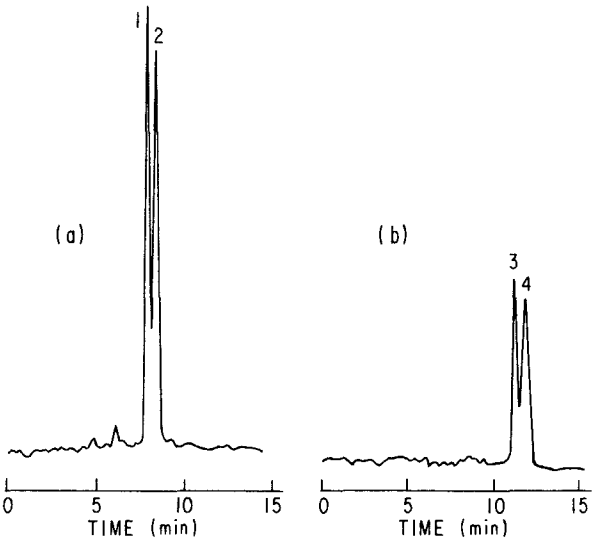

Fig. 4. Separation of closely related uronic acids and lactones. HPX-87-H^+ column, 35°C, 0.6 ml/min. UV detection at 220 nm and 0.5 a.u.f.s. (a) Separation of D-glucuronic acid (1; 7.88 min) and D-mannuronic acid (2; 8.30 min). (b) Separation of D-glucurono-6,3-lactone (3; 11.13 min) and D-mannurono-6,3-lactone (4; 11.71 min). Each acid and lactone present at 2.5 mg/ml.

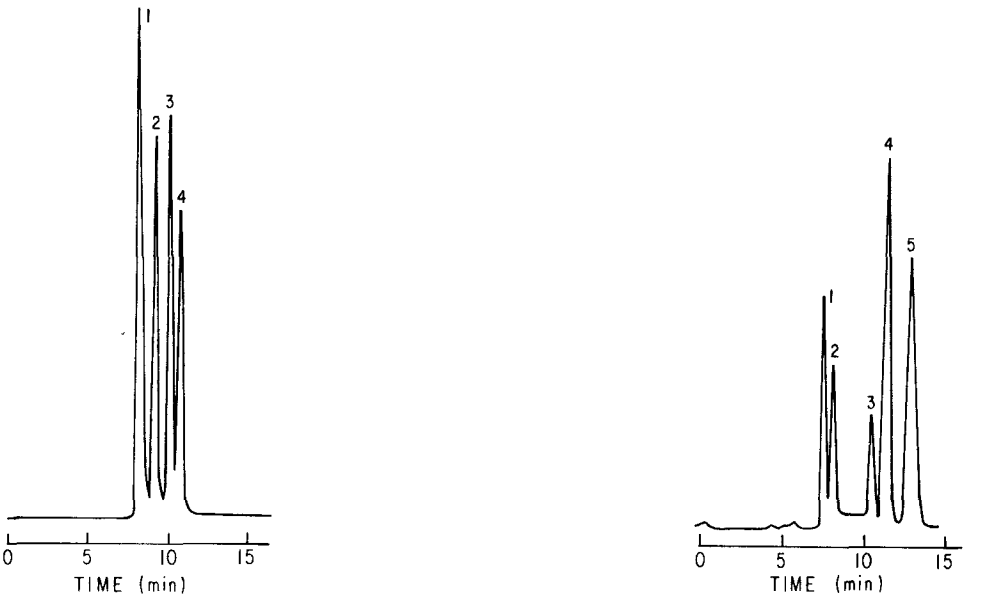

Fig. 5. Separation of closely related lactones. HPX-87-H^+ column at 25°C. UV detection at 220 nm, 0.5 a.u.f.s. Peak identities given in text. Each compound is present in a concentration of *ca.* 6 mg/ml.

Fig. 6. Separation of uronic acids, lactones, and N-acetylated hexosamines. HPX-87-H^+ column at 50°C. UV detection at 220 nm and 0.5 a.u.f.s. Peak identities: 1 = D-glucuronic acid (7.95 min); 2 = D-galacturonic acid (8.51 min); 3 = D-glucurono-6,3-lactone (10.86 min); 4 = 2-acetamido-2-deoxy-D-glucose (11.78 min); 5 = 2-acetamido-2-deoxy-D-galactose (13.53 min). Each compound present at 4.0 mg/ml.

analysis of any, or all of these four compounds in one sample may therefore be readily accomplished. Although baseline separation in these cases is not completely achieved, resolution at this level (R = 0.91 for Fig. 4a and 0.86 for 4b) is adequate for most purposes.

The HPX-87-H^+ column possesses good selectivity for various lactones, as shown in Fig. 5. This complex mixture, containing D-glucono-1,5-lactone (1; 7.94 min), D-glycero-D-guloheptono-1,4-lactone (2; 8.85 min), L-mannono-1,4-lactone (3; 9.80 min), and D-glucurono-6,3-lactone (4; 10.38 min), was separated in less than 12 min.

The importance of column temperature selection is clearly evident in the separation of the mixture of compounds shown in Fig. 6. This mixture, consisting of D-glucuronic acid (1), D-galacturonic acid (2), D-glucurono-6,3-lactone (3), 2-acetamido-2-deoxy-D-glucose (4), and 2-acetamido-2-deoxy-D-galactose (5), is well separated at temperatures near 50°C. From Fig. 1 it is clear that chromatography of this mixture at 25, 35, or 65°C would lead to coeluting peaks, poor resolution, or the destruction of some of the compounds. At 50°C, however, all compounds are well separated with the following resolution values for each pair: D-glucuronic/D-galacturonic acids, 0.95; D-glucurono-6,3-lactone/2-acetamido-2-deoxy-D-glucose, 1.25; 2-acetamido-2-deoxy-D-glucose/2-acetamido-2-deoxy-D-galactose, 1.74. The complete separation of these compounds is useful in the identification and compositional analysis of glycosaminoglycans.

Applications

The use of the HPX-87-H^+ column for the compositional analysis of acidic polysaccharides was studied here. Compositional analysis of polysaccharides is always preceded by a hydrolysis step in which the various monomeric species are liberated. In the case of acidic polysaccharides, the glycuronide-type linkages are difficult to hydrolyze by the normal catalysts, such as mineral acids. Extremely harsh conditions must be used, therefore, to obtain complete hydrolysis of such polymers, and under these conditions, the liberated uronic acids are known[10] to decompose. An alternate method of hydrolysis involves use of purified enzymes.

In this work, a sample of "polygalacturonic acid" was hydrolyzed by a common two-step sulfuric acid-catalyzed procedure and also by a specific enzymatic method. Chromatographic analysis of the sulfuric acid-catalyzed reaction (Fig. 7a)

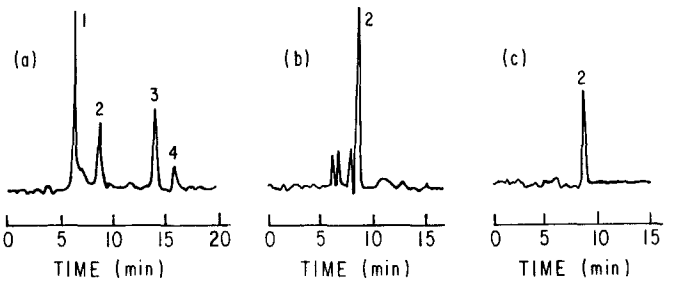

Fig. 7. Hydrolysis of "polygalacturonic acid". HPX-87-H^+ column at 35°C. Other conditions as in Fig. 4. (a) Separation of products from the acid-catalyzed hydrolysis: peak 1, 6.3 min; peak 2, 8.5 min; peak 3, 13.7 min; peak 4, 15.6 min. (b) Enzyme-catalyzed hydrolysis: peak 2, 8.5 min. (c) Standard D-galacturonic acid, 8.5 min.

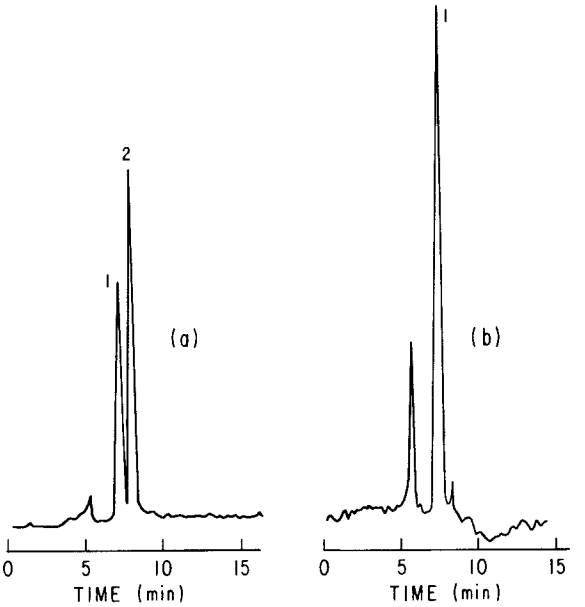

Fig. 8. Analysis of 2-ketogluconic acid produced by rhizosphere bacteria. (a) Standard mixture: peak 1, 2-ketogluconic acid (7.0 min) at 0.7 mg/ml; peak 2, D-gluconic acid (7.7 min) at 1.7 mg/ml. (b) Analysis of bacterial growth culture filtrate: peak 1, 7.1 min, identified as 2-ketogluconic acid (see text).

revealed, as expected, a low yield of D-galacturonic acid (peak 2) and the presence of several major unidentified products (peaks 1, 3, 4). Peaks 3 and 4 probably represent acid-catalyzed uronic acid dehydration or decarboxylation products, as they were also produced in similar hydrolyses of another hexuronate containing polysaccharide, alginic acid. The early peak (1), probably represents excluded, incompletely hydrolyzed polymer.

An identical sample of polygalacturonic acid was then digested with a commercial polygalacturonase-enzyme preparation. In this case (Fig. 7b), the yield of D-galacturonic acid (peak 2) is higher than in Fig. 7a, and the early and late-eluting peaks are either absent or smaller. Analysis of both the acid-catalyzed and enzyme-catalyzed reactions was very rapid with this system since minimal sample cleanup was required and no derivatization was necessary; the total chromatographic analysis time was under 18 min.

D-Gluconic and 2-ketogluconic acids may play important roles in the symbiotic relationship between certain plants and rhizosphere bacteria. The production of these acids by bacteria may cause calcium ions to be sequestered[37], thereby freeing up previously insoluble, and hence, unavailable phosphate for plant use. In this laboratory, "high-acid" producing strains of these organisms were needed for genetic studies. By monitoring (Fig. 8) the acids produced by bacteria in growth-cultures, such strains were readily identified. D-Gluconic and 2-ketogluconic acid could easily be separated (Fig. 8a) and the detection of 2-ketogluconic acid among other bacterial metabolites was readily accomplished (Fig. 8b).

Unlike many silica-based HPLC supports, the polystyrene–divinylbenzene-

type packing in the HPX-87-H^+ column demonstrated excellent stability toward low pH eluents, salts, buffers, and traces of protein contaminants found in samples. Pre-chromatographic clean-up of samples generally consists of filtration, deionization, or at most, the addition of an organic solvent to precipitate sample impurities, followed by filtration. When a precolumn and non-corroding tubing and fittings are properly used, analytical columns of this type can be used for years with very little change in selectivity or efficiency. These properties make this column ideal for the direct determination of uronic and aldonic acids and N-acetylated amino sugars that are present in complex environmental or physiological samples.

In summary, high-performance gel-permeation chromatography on cation-exchange resins is a convenient, sensitive, and selective method for the analysis of carbohydrate-derived acids, lactones, and related compounds. The ability to simultaneously separate and identify lactones and acids is an advantage this system possesses over most anion-exchange or GC methods. Although all 21 of the compounds examined here can not be simultaneously separated, the resolution and selectivity of the system is quite adequate for the separation of many of the acids, lactones, and neutral compounds that would be expected in mixtures such as those demonstrated here. The simple nature, and relatively high capacity of this system also makes it ideal for preparative scale-up. Details of this process will be described elsewhere.

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