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G.L.C. DETERMINATION OF ALDONIC ACIDS AS ACETYLATED ALDONAMIDES

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ABSTRACT

A g.l.c. method for the determination of ribonic, xylonic, mannonic, gluconic, and galactonic acids has been developed. The method utilizes the propensity of aldonic acids to form lactones. The lactones are treated with 1-alkylamines, converted into *N*-(1-alkyl)aldonamides, and acetylated. The peracetylated *N*-(1-alkyl)aldonamides are thermally stable and readily separable by g.l.c. in 13 min on a cyanosilicone column.

INTRODUCTION

Aldonic acids are a biologically and commercially important group of compounds. They appear in sulfite waste pulps, as products from oxidized cellulose, and as metabolic products from a number of microorgnisms, such as *Aspergillus niger*. Gluconic acid is used nutritionally as a carrier for calcium, zinc, and iron and, commercially, as a sequestering agent for a number of divalent and trivalent metal ions.

Analytical methods for their detection and quantitation have covered a wide spectrum of techniques. Paper chromatography[1], thin-layer chromatography[2], gas–liquid chromatography[3,4], and high-performance liquid chromatography[5] have been used to analyze samples containing aldonic acids. Some of the methods cited require long development-times, lack resolution or sensitivity, or are difficult to quantify. Every procedure is complicated by the tendency of a pure aldonic acid to form mixtures consisting of the 1,4- and 1,5-lactones in addition to the free acid[6]. The relative amounts of each component vary with the specific acid temperature, concentration, solvent, pH, and time. Under certain circumstances, the equilibrium may be driven toward formation of a 1,4-lactone. When a mixture containing a 1,4-lactone, a 1,5-lactone, the corresponding aldonic acid, and 2M hydrochloric acid is evaporated to dryness, only the 1,4-lactone is isolated[7]. However, evaporation of

*The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

solutions containing hydrochloric acid results in the formation of a constant boiling azeotrope containing 20.2% of HCl. Consequently, if the mixture contained any acid-labile components, they might be degraded by the hydrochloric acid solution. In the method developed, mixtures of 1,4- and 1,5-lactones[8] are not a problem. Both are converted to the same *N*-alkylaldonamide.

EXPERIMENTAL

Materials. — Pyrrolidine, 1-propylamine, 1-butylamine, and 1-pentylamine were obtained from Aldrich Chemical Co. D-Mannitol and L-mannono-1,4-lactone were obtained from Sigma Chemical Co., and sodium D-gluconate, D-galactono-1,4-lactone and D-glucono-1,5-lactone and α-D-glucose pentaacetate were obtained from Pfanstiehl Laboratories, Inc. G.l.c. coated supports were obtained from Supelco (3% SP-2340 on 100–120 mesh Supelcoport) and Applied Science (3% JXR on 100–120 mesh Gas Chrom Q).

G.l.c. analysis. — G.l.c. analysis was performed on a Packard Instrument Model 428 gas chromatograph equipped with dual f.i.d. and dual electrometers. Trimethylsilyl (Me_3Si) derivatives were analyzed on a stainless-steel column (2.45 m × 3.2 mm o.d.) packed with 3% JXR on Gas Chrom Q 100–120 mesh. The temperature was programmed from 150 to 200° at a rate of 2°/min and the helium flow was 20 mL/min. The acetate derivatives were analyzed on a glass column (1 m × 2 mm i.d.) packed with 3% SP-2340. The temperature was programmed from 220 to 250° at a rate of 32°/min after an initial delay of 2 min. The helium flow-rate was 20 mL/min. For quantitation, the temperature was programmed from 190 to 250° at a rate of 5°/min.

Quantitation. — Calibration-curve (plot of relative detector response *vs.* mg sample) determination for *N*-(1-propyl)-, *N*-(1-butyl)-, *N*-(1-pentyl)-, *N*-(1-hexyl)-, and *N*-pyrrolidino-, L-mannonamides, D-gluconamides, and D-galactonamides used *myo*-inositol as the internal standard. Triplicate samples of L-mannono-1,4-lactone, D-glucono-1,5-lactone, and D-galactono-1,4-lactone, at 4 concentration levels (1–7 mg) containing 3 mg of *myo*-inositol were treated as described in the Experimental section with each of the amines. G.l.c. analyses of the peracetylated amides and *myo*-inositol were performed as described. Electrometer sensitivity was set at range 10, attenuation at 32. Detector response (peak area) was calculated by using a Modcomp (Modular Computer Systems) computer. A plot of relative detector response (detector response of aldonamide/detector response of 3 mg of *myo*-inositol) *vs.* mg of aldonic acid was linear. The average corelation-coefficients for all of the samples was 0.998, with a standard deviation of ±0.001. The *K* factors for the *N*-(1-propyl)aldonamides were: L-mannono 0.97, D-glucono 0.98, and D-galactono 0.92.

ACETYLATED *N*-ALKYLALDONAMIDES

N-*(1-Propyl)aldonamides.* — A mixture of lactones (1–5 mg of each, mannono-1,4-lactone, glucono-1,5-lactone, and galactono-1,4-lactone) in a 16 × 125 mm culture tube was dried *in vacuo* for 2 h at 80° to ensure that the whole sample was in the form of the lactone. A 1:1 mixture (by volume) of dry pyridine and 1-propylamine was added (0.5 mL), and the tube was sealed with a Teflon-lined screw-cap. The mixture was heated in an aluminum heating-block, with shaking to ensure complete solution, for 30 min at 50°. The cap was removed and dry nitrogen was bubbled through the heated solution for ~20 min until a white solid residue remained. Pyridine (0.5 mL) and acetic anhydride (0.5 mL) were added, and the mixture was occasionally shaken while being heated in an aluminum heating-block for 40 min at 90°. The sample is suitable for g.l.c. When smaller quantities of lactone are used, it is advisable to remove the pyridine and acetic anhydride by bubbling nitrogen through the heated solution (50°) and then reconstituting with 100 μL of dry acetone or dichloromethane.

N-*(1-Butyl)aldonamide.* — As preceeding, except the temperature of reaction and evaporation was raised to 60°.

N-*(1-Pentyl)aldonamide.* — As preceeding, except the temperature of reaction and evaporation was raised to 65°.

N-*Pyrrolidinaldonamide.* — As preceeding, except the temperature of reaction and evaporation was raised to 65° and the time of reaction was lengthened to 45 min.

Optimization of lactone formation. — A solution (2 mL) containing 21.47 mg/mL of sodium D-gluconate was poured onto a column prepared from a Pasteur pipet and filled with 2 mL of Bio-Rad AG 50W-X8 200–400 mesh cation-exchange resin in the acid form. The gluconate was eluted from the column with 11 mL of water. The total volume was adjusted to 12 mL. Portions (1 mL) were transferred to 16 × 125-mm culture tubes and evaporated *in vacuo* (vacuum pump with Dry Ice trap) to dryness at 40, 60, 70, and 85° in a Büchler Vortex Evaporator. Samples were periodically removed, converted into their Me_3Si derivatives[9], and chromatographed on the 3% JXR column.

RESULTS AND DISCUSSION

The method developed takes advantage of the ease with which aldonic acids form lactones[8]. The lactones are quantitatively converted into *N*-(1-alkyl)-aldonamides by treatment with a primary amine in an aprotic solvent. Both the 1,4- and 1,5-lactones form the same *N*-(1-alkyl)aldonamide. Acetylation with acetic anhydride gives the heat-stable, peracetylated *N*-(1-alkyl)aldonamide, which may be chromatographed by g.l.c. on a cyanosilicone column. Ribonic, xylonic, mannonic, gluconic, and galactonic acids were treated as described and were readily separated by g.l.c. in 13 min. Glucose, when treated as described, forms an *N*-(1-

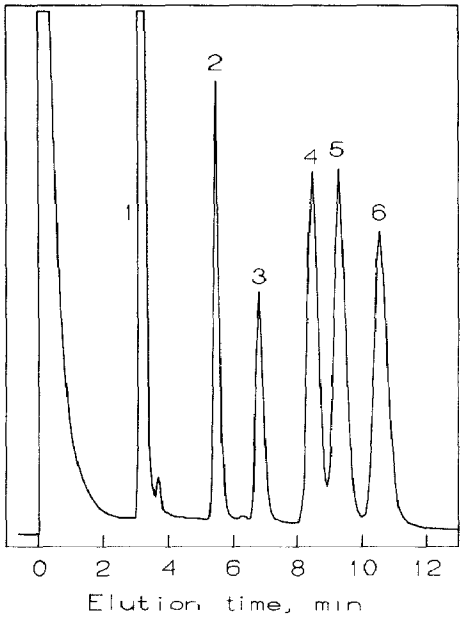

Fig. 1. Separation of *N*-propylaldonamide acetates by gas–liquid chromatography on an SP-2340-packed glas column. The temperature was kept for 2 min at 220° and then increased to 250° at 32°/min. 1, Mannitol hexaacetate; 2, *N*-propylribonamide acetate; 3, *N*-propylxylonamide acetate; 4, *N*-propylmannonamide acetate; 5, *N*-propylgluconamide acetate; 6, *N*-propylgalactonamide acetate.

TABLE I

G.L C RETENTION TIMES FOR ALDONAMIDES ON SP-2340[a]

Aldonic acid	*Amide*[b]			
	N-*Propyl*	N-*Butyl*	N-*Pentyl*	N-*Pyrrolidinyl*
D-Ribono	5.54			
D-Xylono	6.73			
L-Mannono	8.53	8.91	9.53	12.09
D-Glucono	9.34	9.78	10.53	10.16
D-Galactono	10.66	11.26	12.08	8.96

[a]G.l.c. conditions were: 220° held for 2 min, and then temperature-programmed at 32°/min to 250°.
[b]Mannitol hexaacetate was used as the internal standard and had a retention time of 3.10 min.

alkyl)glucosylamine peracetate that is separable from the *N*-(1-alkyl)aldonamide prepared from gluconic acid. The structure of *N*-(1-butyl)glucosylamine was confirmed by g.l.c.–m.s. Chemical ionization with isobutane gave a strong molecular ion (MH^+) at 446.

The *N*-(1-alkyl)aldonamides are a readily prepared and versatile set of derivatives for the detection and quantitation of aldonic acids. They preserve the symmetry of the molecule, which would be lost if a modified alditol acetate proce-

TABLE II

PERCENT OF LACTONE AND ACID PRESENT AFTER VORTEX EVAPORATION OF GLUCONIC ACID SOLUTION

Temperature	*Component*	*Time of evaporation*		
		40 min	*60 min*	*120 min*
40°	Lactone		81.2%	89.2%
	Acid		18.8%	10.8%
60°	Lactone		94.8%	98.8%
	Acid		5.2%	1.2%
70°	Lactone	96.4%	98.3%	99.5%
	Acid	3.6%	1.7%	0.5%
85°	Lactone	99.4%	99.9%	
	Acid	0.6%	0.1%	

dure were employed. Each aldonic acid forms one *N*-(1-alkyl)aldonamide unlike the lactone, which can be present in two different forms. In addition, if a complex mixture is being analyzed and the aldonamide peak coincides with or partially overlaps another peak in the chromatogram, its relative location can readily be changed by changing the *N*-alkyl component of the aldonamide. For example, if the *N*-(1-propyl)aldonamide overlaps some other peak in the chromatogram, then another portion of the sample can be converted into the *N*-(1-butyl)- or *N*-(1-pentyl)-aldonamides, which have longer retention-times. This procedure should be easier, less time-consuming, and less expensive than the trial-and-error method of testing a wide variety of columns.

Five aldonamides were considered (L-mannono, D-glucono, D-galactono, D-xylono, and D-ribono). The *N*-(1-alkyl) components considered were the 1-propyl, 1-butyl, 1-pentyl, and pyrrolidinyl. A typical separation is shown in Fig. 1. Peak 1 (mannitol hexaacetate) was used as the internal standard.

For routine qualitative identification, fast elution-times are obtained by programming from 220 to 250° at 32°/min. A summary of retention times for the *N*-(1-propyl)-, *N*-(1-butyl)-, *N*-(1-pentyl)-, and *N*-pyrrolidinyl series is incorporated in Table I.

Aldonic acids and lactones may epimerize if subjected to alkaline conditions. Inasmuch as primary amines are quite alkaline, it was necessary to check out this possibility. Pure D-glucono-1,5-lactone, D-galactono-1,4-lactone, D-ribonolactone, D-xylonolactone, and L-mannono-1,4-lactone were treated with 1-propylamine according to the described protocol. Only one peak appeared from each lactone. Gluconic acid did not epimerize to mannonic acid nor did mannonic acid epimerize to gluconic acid.

Aldonolactones may form dehydrated products when treated with pyridine and acetic anhydrides[10]. Such dehydration does not occur with the *N*-(1-alkyl)-aldonamides under the conditions studied. This was confirmed by a g.l.c.–mass spectrum of the peak obtained from *N*-butylgluconamide. Chemical ionization with

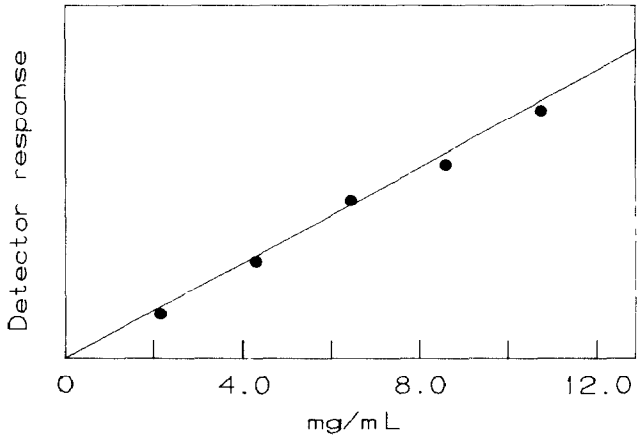

Fig. 2. A plot of relative detector response *vs.* mg of gluconic acid converted into *N*-propylgluconamide acetate using acetylated D-glucitol as the internal standard.

isobutane gave a molecular ion, MH^+, at 462.

It is essential that all of the aldonic acid be converted into the lactone. It is immaterial whether it forms the 1,4- or 1,5-lactone, inasmuch as both forms readily undergo nucleophilic substitution by amines. A model system was set up to determine what experimental conditions were necessary to ensure that all of the aldonic acid was in the lactone form. The readily available sodium gluconate was passed through a cation-exchange column (H^+ form) and thus converted into gluconic acid. Solutions were evaporated at 40, 60, 70, and 85° in a Büchler vortex evaporator. Residues were then converted into Me_3Si derivatives and chromatographed on the JXR column. Retention times for the lactones were 15.7 (1,4) and 16.3 (1,5) min, whereas that of the acid was 21.3 min. Evaporation at 40° (22 min) gives a mixture containing 29.2% acid and 70.8% lactone. A summary of the results obtained at different temperatures is found in Table II. It is apparent that evaporation *in vacuo* at 85° for a period of 1 h is sufficient to convert 99.9% of the acid into the lactone. If a lower temperature is selected, a proportionally longer time-period will be required.

The amines readily react with the lactones to form the *N*-alkylaldonamides. The amine and the pyridine must be kept dry. Traces of moisture hydrolyze the lactone and little if any amide results. The vortex evaporator with the cover off was used as a convenient heated shaker for conducting the reaction. Solution usually occurs within 2–3 min. Evaporation of the amines is usually completed within 10 min if a fast flow of dry nitrogen is used. The acetylation occurs with some color formation. The tubes vary from colorless to light amber. The *N*-pyrrolidine compounds are the most deeply colored. The samples are stable for reasonable time-periods, namely, weeks.

To determine whether or not the g.l.c. response was linear over a reasonable range, a series of solutions was made with *myo*-inositol (D-mannitol, D-glucitol,

β-D-glucose pentaacetate, or phenyl β-D-glucopyranoside could have been used) as the internal standard. The *N*-(1-butyl)-, *N*-(1-pentyl)-, *N*-(1-hexyl)-, and *N*-(1-propyl)-amides of gluconic, mannonic, and galactonic acids were prepared as described. The *N*-(1-alkyl)aldonamide acetates and *myo*-inositol acetate were temperature-programmed from 190 to 260° at 5°/min. A summary of a series is presented in Fig. 2.

The elution patterns for the series are as expected (an analogy to the aldononitrile acetate series), namely, manno-, gluco-, galacto-; except for the pyrrolidino series, in which it is reversed, that is, galacto-, gluco-, and manno-. This change might afford some advantage in a situation where overlapping peaks are present.

The g.l.c. method described uses the simply and rapidly prepared *N*-(1-alkyl)amide derivatives of the aldonic acids. They are readily separable by g.l.c. in a short period of time. The detector response of the *N*-alkyl derivatives is proportional to the amount of aldonic acid in the original solution. Mixtures containing glucose in addition to aldonic acids can readily be analyzed by this method.

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