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DETERMINATION OF D-GLUCONIC, 5-KETO-D-GLUCONIC, 2-KETO-D-GLUCONIC AND 2,5-DIKETO-D-GLUCONIC ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The application of high-performance liquid chromatography to the quantitative analysis of D-gluconic, 5-keto-D-gluconic, 2-keto-D-gluconic and 2,5-diketo-D-gluconic acids in biological fluids is described. Separation of these acids is achieved on a column packed with Aminex A-28 anion-exchange resin, using ammonium formate as the eluent. Samples are filtered through a 0.45-μm membrane and injected directly onto the column. Quantitation of gluconic acid in acidic solutions requires hydrolysis of the gluconolactones before injection.

INTRODUCTION

D-Gluconic, 5-keto-gluconic (D-*xylo*-5-hexulosonic), 2-keto-D-gluconic (D-*arabino*-2-hexulosonic) and 2,5-diketo-D-gluconic (D-*threo*-2,5-hexodiulosonic) acids are produced by biochemical and catalytic oxidation of glucose. Currently gluconic acid is either determined enzymatically[1] or by high-performance liquid chromatography (HPLC)[2]. The HPLC determination of gluconic acid in wine produced from botrytised grapes has been recently reviewed[3].

After conversion of glucose to gluconic acid, some bacteria carry the oxidation further to yield ketogluconic acids. Production of these acids is important in the classification of acetic acid bacteria and qualitative screening is carried out routinely by thin-layer chromatography[4]. This method requires three developments and is time-consuming, although spraying with *o*-phenylenediamine does give specific colour reactions with these acids.

Little has been published on the HPLC determination of ketogluconic acids. Separation and quantitation of gluconic and 2-keto-D-gluconic (2KG) acids has been achieved on a carbohydrate column[5] and Aminex anion-exchange resins have also been used with some success. Aminex A-25 has been used to determine the homogeneity of 2,5-diketo-D-gluconic (25DKG) acid, prepared by bacterial fermentation

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and ion-exclusion chromatography[6]. Aminex A-28 separates gluconic from ketogluconic acids but the latter were not well-resolved and not quantitated[2].

A recent report by Sonoyama *et al.*[1] emphasizes the time-consuming nature of methods used to determine gluconic and ketogluconic acids. These methods included gas and paper chromatography, spectrophotometric and enzymic analyses. The present study describes a simple quantitative HPLC method on Aminex A-28 which can separate gluconic, 5-keto-D-gluconic (5KG), 2KG and 25DKG simultaneously in biological samples without prior isolation or derivatization.

EXPERIMENTAL AND RESULTS

Apparatus

The liquid chromatograph used consisted of a Waters Assoc. M6000A pump, R401 refractive index monitor, U6K injector and a Hewlett-Packard 3390A integrator.

The separation was carried out on a 300 × 4 mm I.D. stainless-steel column, slurry-packed with Aminex A-28 resin (formate), particle size 9 ± 2 μm (Bio-Rad Labs.). The column was covered with an aluminium jacket which was heated by water circulated from a 60 ± 1°C bath. A guard column (Waters Assoc.), manually packed with the same resin was also used. The eluent consisted of 0.3 *M* formic acid adjusted to pH 3.75 with ammonia. Flow-rate was 0.5 ml/min and the initial column pressure was 12.4 MPa.

Reagents

The potassium salts of D-gluconic acid and 5KG, and the calcium salt of 2KG were purchased from Sigma. The calcium salt of 25DKG was prepared by fermentation of glucose with *Acetobacter fragum* ATCC 21409[7]. Other reagents used were analytical grade.

Purification of calcium 25DKG

Production of calcium 25DKG was monitored by HPLC (Fig. 1). Cultures were clarified by centrifugation and lyophilized portions of this material (1–2 g) redissolved in water, were decolorized with carbon (Norit) and filtered. This solution was then chromatographed on a column (400 × 30 mm I.D.) of AG50W-X8 (50–100 mesh) resin in the calcium form (Bio-Rad Labs.) and eluted with water. The elution profile was monitored by HPLC and selected fractions were lyophilized to give a white amorphous calcium salt; $[\alpha]_D^{20}$ −50.5° (cl, water); literature $[\alpha]_D^{23}$ −51.1 ± 5° (ref. 8) and $[\alpha]_D^{23}$ − 52.5° (ref. 6) (cl, water). Elemental analysis calculated for $(C_6H_7O_7)_2Ca \cdot 3H_2O$: C, 30.25; H, 4.24. Found C, 30.16; H, 4.58.

A bis-2,4-dinitrophenylhydrazone (DNP) derivative prepared as described by Wakisaka[8], and recrystallized three times from ethyl acetate, gave the uncorrected melting point of 154–156°C (dec.) previously obtained by Stroshane and Perlman[9].

The detailed IR spectrum obtained by Bernaerts and De Ley[10] for calcium 25DKG produced by *Chrombacteriunm lividum* could not be reproduced using KBr discs of the above salt. However, the spectrum obtained from the DNP derivative was very similar to that published elsewhere[8].

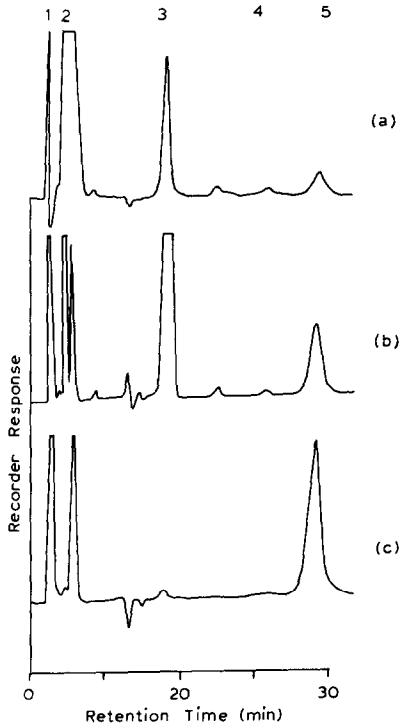

Fig. 1. HPLC profiles of the fermentation of glucose by *A. fragum* at (a) 12, (b) 18 and (c) 24 h. (1) cations; (2) glucose; (3) gluconate; (4) 2KG; (5) 25DKG (25-μl injection).

HPLC separation and standardization

The column was equilibrated to 0.3 *M* formic acid adjusted to various pH values with ammonia. Injections of *ca.* 1% standard solutions of potassium gluconate, potassium 5KG and calcium 2KG were made at pH 3.50, 3.75, 4.00, 4.50, 4.75 and 8.00 (Fig. 2). Baseline separation was achieved at pH 3.75 for gluconate, 5KG, 2KG and 25DKG (Fig. 3) and several injections of each individual standard at three different concentrations between 0.1–1.0% were made to determine reproducibility and linearity (Table I).

Several other acids, dissolved in water, were injected individually with potassium gluconate (*ca.* 0.2%), to determine relative retention times and possible interference with gluconate, 5KG, 2KG and 25DKG (Table II).

While glutaric and succinic acids gave relative retention times consistent with the time scale of the analysis for the gluconic acid derivatives it was noted that oxalic, fumaric and aconitic acids had very long retention times. They were measurable by monitoring UV absorption but gave distorted peak shapes. Refractive index detection yielded broad flat peaks which could only interfere with the analytical method if present in large concentrations (Fig. 4).

Examination of this figure reveals the presence of a peak in the region 0.73–0.76 relative retention time. It was noted that injection of acids in water or as their ammonium salts in eluent produced negative responses in this region of the chro-

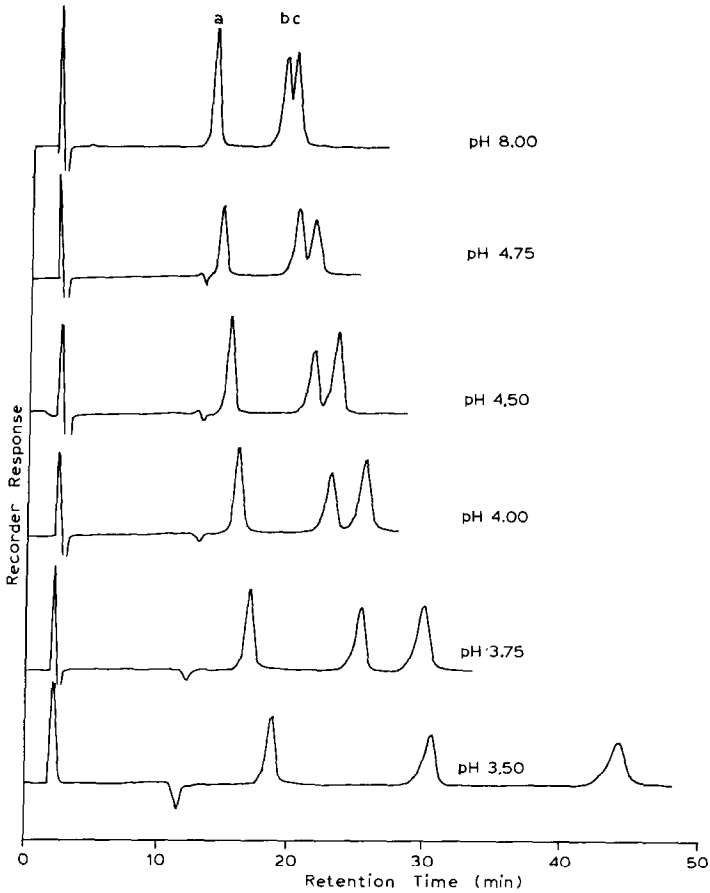

Fig. 2. HPLC profiles showing the effect of pH on the retention times of (a) gluconate; (b) 5KG; (c) 2KG (25-μl injection).

matogram while free acid dissolved in eluent produced a positive peak. This is attributed to the production of vacant peaks[11] arising from sensitivity in the detectors to variation in pH in the eluent; strong acids (low pK_{a_1}) produce positive artifacts as the eluent pH is reduced.

The effect of column temperature on the peak areas of a mixed standard solution of potassium gluconate, calcium 2KG, calcium 25DKG and a separate solution of potassium 5KG was determined (Fig. 5). Potassium 5KG standards were analysed individually or added to a mixed standard just prior to injection. Calcium 5KG is formed on addition and this is sparingly soluble and precipitates out of solution on standing.

Quantitation of gluconic acid

Quantitation of gluconic acid in acidic solutions required hydrolysis of the gluconolactones by adjustment to pH 9 with sodium hydroxide. To demonstrate the formation and hydrolysis of lactones, a *ca.* 1% solution of gluconic acid was prepared

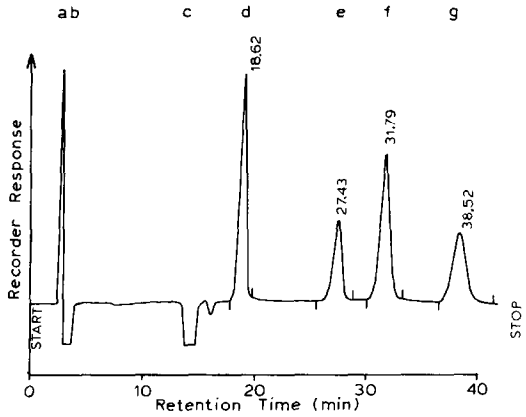

Fig. 3. HPLC profile of a standard solution, (a) cations; (b) first and (c) second negative peak; (d) gluconate; (e) 5KG; (f) 2KG; (g) 25DKG (100-μl injection).

by treatment of potassium gluconate with Amberlite IR-120(H) resin. This solution was allowed to equilibrate for 48 h at 20°C. A small amount of lactone was present when analyzed by HPLC (Fig. 6a). Complete hydrolysis was achieved after 1 h at pH 9, increasing the gluconate area by 4.8% (Fig. 6b).

Sample preparation

All standard solutions and biological samples such as fermentation fluids, were filtered through a 0.45-μm acrodisc filter (Gelman) and either 25 μl (25–250 μg acid) or 100 μl (100–1000 μg acid) injected onto the column.

TABLE I

PERCENTAGE RELATIVE STANDARD DEVIATION (RSD) OF STANDARDS

Standard	*Acid (%, w/v)*	*n*	*R.S.D. (%)*
K-gluconate	0.64	8	1.2
	0.40	8	1.5
	0.16	8	3.5
K-5KG	1.00	5	0.9
	0.42	5	1.4
	0.17	5	2.2
Ca-2KG	1.04	5	0.2
	0.57	5	0.5
	0.12	5	3.5
Ca-25DKG	0.94	6	4.6
	0.51	6	3.7
	0.12	6	4.1

TABLE II

RELATIVE RETENTION TIMES (RRT) OF GLUCONOLACTONES AND SOME ACIDS RELATIVE TO GLUCONIC ACID

Acid	*RRT*
Glucono-1,5-lactone	0.29
Glucono-1,4-lactone	0.45
Kojic	0.67
Gluconic	1.00
Galactonic	1.01
Glycollic	1.38
Glucuronic	1.38
Lactic	1.44
5KG	1.47
2KG	1.71
25DKG	2.07
Phosphoric	2.46
Glutaric	2.80
Succinic	3.01
Oxalic	19
Fumaric	26
Aconitic	98

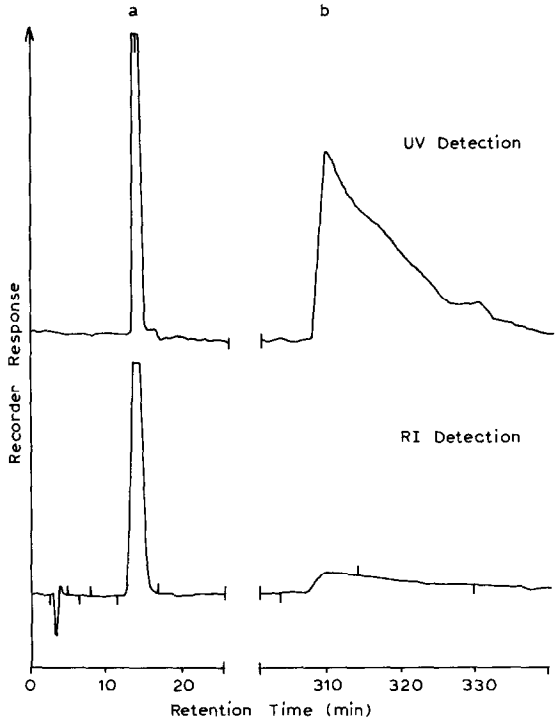

Fig. 4. Comparison of HPLC profiles obtained from UV and refractive index (RI) detection of oxalic acid (100 μl of *ca.* 2% dissolved in eluent). (a) Positive peak and (b) oxalate. The UV monitor used was a Waters Assoc. 450 variable-wavelength detector set at 240 nm and 0.1 a.u.f.s.

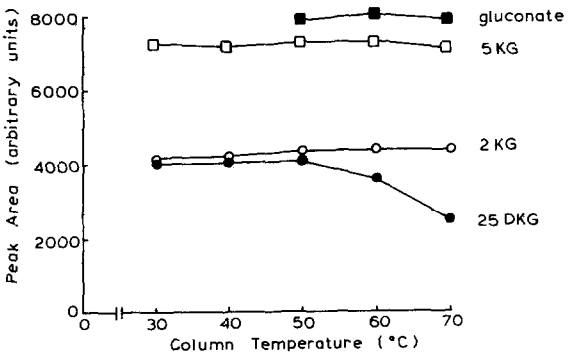

Fig. 5. Effect of column temperature on peak area. Concentrations: gluconate and 5KG, 166 μg/100 μl; 2KG and 25DKG, 95 μg/100 μl.

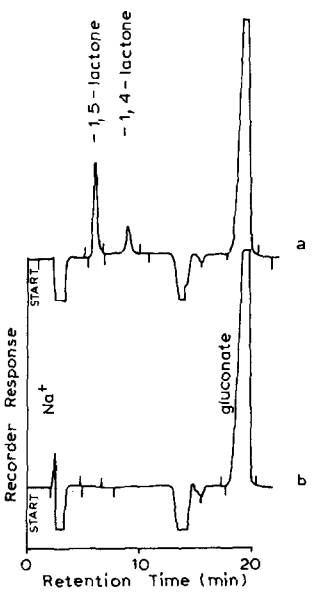

Fig. 6. (a) Formation and (b) hydrolysis of gluconolactones.

DISCUSSION

The results show that gluconic, 5KG, 2KG and 25DKG acids can be separated by the HPLC system described. This method is particularly suited to the monitoring of fermentation fluids such as those of acetic acid bacteria (Fig. 1).

Changing the pH of the eluent has a dramatic effect on the resolving power of the column (Fig. 2). At pH 3.75 baseline separation of all four acids was achieved (Fig. 3) and this pH was used for all subsequent analyses. Eluent strength had a direct effect on retention times but did not improve resolution. A five-fold decrease in eluent strength from 0.5 *M* to 0.1 *M*, resulted in a five-fold increase in retention time. An eluent strength (0.3 *M*) was chosen which separated gluconate and the

second negative peak (Fig. 3). This allows electronic integration without giving unduly long retention times.

Although a column temperature of 60°C was used for the development of this method, it was found that there is some on-column degradation of 25DKG above 50°C (Fig. 5). Gluconate, 5KG and 2KG are stable up to 70°C with little change in peak area. Above 50°C the degradation of 25DKG becomes significant with a 12% loss of area between 50 and 60°C. The rate of degradation accelerates to a further 31% loss between 60 and 70°C. The lower the column temperature, the closer gluconate and the second negative peak elute. Running the column at 50°C would require lowering the pH to 3.50 so that adequate resolution is maintained and quantitation of gluconate can be achieved.

Standard curves for gluconate, 5KG, 2KG and 25DKG were linear between 0.1 and 1.0%. Linearity outside this range was not investigated. Relative standard deviation (R.S.D.) values (Table I) were obtained over a few days with several eluent changes and except for 25DKG, R.S.D. generally increased with decreasing salt concentration. The average R.S.D. for 25DKG was 4.1% and the increased variation obtained for this compound was undoubtedly due to thermal degradation on the column at 60°C.

The method described here is not suitable for the separation of similar aldonic acids. Table II shows that galactonic and gluconic acids are not separable. Separation of these acids on anion-exchange resins has been reviewed[12]. Glycollic, glucoronic and lactic acids all have similar relative retention times to 5KG and where these are present, further pH adjustment may be necessary to improve resolution.

The bacterial formation[13], hydrolysis[14], and conformation[15] of glucunolactones have been studied in detail elsewhere. On the HPLC system described here, the lactones are well separated from gluconic acid (Fig. 6) and therefore need to be hydrolyzed if quantitation is required. The amount of lactone present at any one time varies and depends on the solvent, pH, temperature and concentration. A period of 3 h at pH 9[16] or 4 h at pH 8.5[17] has been shown to be sufficient for the complete hydrolysis of gluconolactones. Determination of 25DKG is necessary before any pH adjustment is made as it is unstable, especially above pH 4.5 (ref. 18). Conformation of ketogluconic acids in solution has been studied by ^{1}H- and ^{13}C-NMR spectroscopy and lactone formation by these acids has not been detected[19]. Addition of calcium carbonate[7] or phosphate buffer[10] has been used to control pH during fermentation and to prevent acidification.

Acetic acid bacteria produce pigments which sorb onto the top of the guard column. Manual replacement of the first 1–2 mm of resin at the first sign of any pressure rise will increase column life. During a twelve months period over 1000 injections (25–100 μl) were made onto the column. Column pressure gradually increased from 12.4 to 27.6 MPa during this time. The column was then repacked with fresh resin and this restored initial running conditions. Without recirculation of eluent, over 500 × 100 μl injections have already been made on this column and no increase in pressure (12.4 MPa) or decrease in resolution has been observed.

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