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Note

Determination of D-glucaric acid by high-performance liquid chromatography

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In 1963 Marsh [1] established D-glucaric acid (GA) to be a normal constituent of human urine and demonstrated an oxidative metabolic pathway of D-glucuronolactone in mammals.

Not only can hepatic cytochrome *P*-450 mixed function oxidase enzyme systems be induced, but many compounds have an inducing effect on the formation of glucuronic acid from glucose and galactose, too [2—6]. The amount of urinary D-glucaric acid seems to correlate with the amount of enzyme-inducing drugs [3, 5, 7]. Many drugs, environmental chemicals and food additives can induce hepatic microsomal enzyme activities [4, 8—11]. The daily urinary excretion of GA has been applied as a useful test to assess the hepatic microsomal drug-metabolizing enzyme activity [10, 12—14] and the enzyme induction by xenobiotics as well [15].

The methods most used for the quantitative determination of GA in urine are still the enzymic assays modified from the method of Marsh [1], which are based on the β-glucuronidase inhibitory effects of boiled solutions of GA, but the results may vary, since many factors in the assay itself may influence the β-glucuronidase activity [16]. Ishidate et al. [17] in 1965 published an ion-exchange chromatographic method, which was modified by Tokola et al. [18] in 1975. Some gas—liquid chromatographic methods for lactone-forming organic acids have also been introduced. In all of them a volatile derivative of the acid is formed. The carboxyl groups are usually converted into methyl esters or trimethylsilyl esters, or the hydroxyl groups into trimethylsilyl ethers [16, 19—22].

The separation of GA and its lactones from aqeuous solution by high-performance liquid chromatography (HPLC) is presented in this paper. Some possibilities for the analysis of GA in urine are also investigated.

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EXPERIMENTAL

Apparatus

A Waters Model 6000 A liquid chromatography pump was used together with a Waters Lambda-Max Model 480 LC spectrophotometer detector and injector (Waters Assoc., Milford, MA, U.S.A.) and a Goerz Servogor S recorder (Goerz Electro, Vienna, Austria). The column was a self-packed Spherisorb-NH_2, particle size 5 μm (228 × 5 mm I.D.).

Reagents

D-Saccharic acid monopotassium salt (Sigma No. S-0250), D-saccharic acid 1,4-lactone (Sigma No. S-0375) and glycolic acid (Sigma No G-1884) were all from Sigma (St. Louis, MO, U.S.A.). Acetonitrile, HPLC-grade, was from Rathburn Chemicals (Walkerburn, U.K.). Orthophosphoric acid and 1-octanol were from E. Merck (Darmstadt, F.R.G.), absolute ethanol was from Oy Alko (Rajamäki, Finland).

Urine samples

Twenty-four-hour urine samples for glucaric acid were collected from a healthy female subject.

Sample preparation

Under acidic and neutral conditions GA is always in equilibrium with its intramolecular esters, the lactones, including D-glucaro-1,4-lactone, D-glucaro-3,6-lactone and D-glucaro-1,4-3,6-dilactone [18]. The equilibrium depends on pH and temperature [15]. In basic solution (pH $\leqslant$ 8) GA is totally in the form of free acid.

Aqueous standards (in concentrations of 0.5—3.0 mg/ml) were made alkaline by addition of 1 *M* sodium hydroxide to pH 8. Aqueous standards and urine samples were made acidic by 1 *M* hydrochloric acid to pH 2.5.

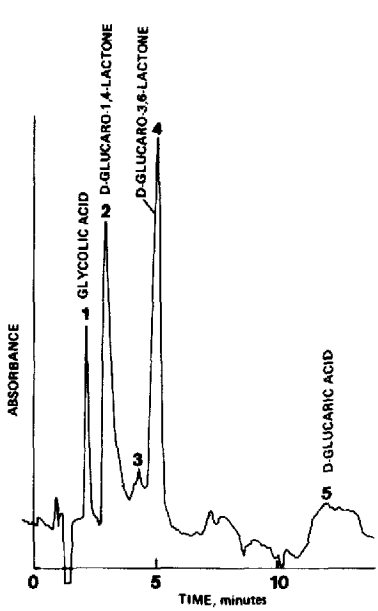
Urine samples and standards were hydrolyzed by boiling in water for 1 h, cooled to room temperature and centrifuged (2000 *g* for 5 min). Glycolic acid was used as internal standard in a final concentration of 0.5 mg/ml. Urine samples were concentrated by vacuum distillation to 1/10 of the volume. To prevent foaming a few drops of 1-octanol were added.

HPLC procedure

The mobile phase was 0.01 *M* phosphoric acid (pH 2.5)—acetonitrile (75:25, v/v) and the flow-rate 2 ml/min at room temperature. The mobile phase was filtered through a Millex-HV filter unit, 0.45 μm (Millipore) and degassed ultrasonically (Bransonic 220). It took several hours to stabilize the column with the buffer—acetonitrile eluant, so the column was eluted at a minimum flow-rate overnight. Standards and concentrated urine samples were injected into the column. The eluate was monitored at 220 nm.

RESULTS

Fig. 1 shows the HPLC separation of GA and its lactones at pH 2.5 with the



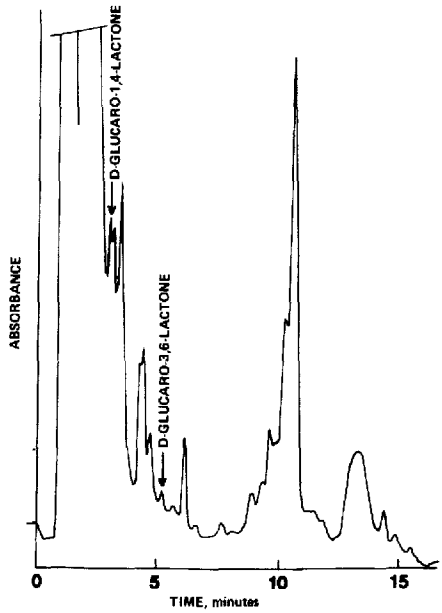


Fig. 1. HPLC separation of D-glucaric acid from its lactones after hydrolysis at 100°C and pH 2.5 for 1 h. Glycolic acid was used as the internal standard. Conditions: column Spherisorb-NH_2; mobile phase acetonitrile—0.01 *M* phosphoric acid (25:75, v/v); ultraviolet detection at 220 nm; flow-rate 2 ml/min; 0.01 a.u.f.s; chart speed 600 mm/min.

Fig. 2. HPLC chromatogram of concentrated normal human urine by ultraviolet detection (220 nm). Conditions as in Fig. 1.

Spherisorb-NH_2 column used in the weak anion-exchange mode. The internal standard (glycolic acid) elutes first (peak 1), and next D-glucaro-1,4-lactone (peak 2), which is well separated from the internal standard. It was identified with a pure D-glucaro-1,4-lactone solution. A small peak 3, obviously D-glucaro-1,4-3,6-dilactone, is not totally separated from peak 4, obviously D-glucaro-3,6-lactone (according to Fiedler et al. [16]). D-Glucaric acid elutes last. The separation of all compounds takes less than 13 min.

Table I summarizes the chromatographic behavior and the composition by area (%) of GA and its lactones and the internal standard glycolic acid.

TABLE I

HPLC EVALUATION OF SEPARATED COMPOUNDS IN D-GLUCARIC ACID ANALYSIS ON SPHERISORB-NH_2 COLUMN AT pH 2.5

Compound	Retention time (min)	Capacity factor (k')	Composition by area (%)
Glycolic acid	2.1	0.50	—
D-Glucaro-1,4-lactone	2.9	1.07	30
Unknown	4.4	2.14	5
D-Glucaro-3,6-lactone	5.1	2.64	40
D-Glucaric acid	12.1	7.64	25

TABLE II

THE PRECISION OF A SERIES OF D-GLUCARIC ACID SAMPLES AT A CONCENTRATION OF 2 mg/ml

	D-Glucaro-1,4-lactone/ internal standard	D-Glucaro-3,6-lactone/ internal standard
	1.77	2.66
	2.20	2.70
	2.05	2.72
	1.95	2.79
	2.21	2.77
Mean	2.04	2.73
S.D.	0.18	0.05
C.V. (%)	9.0	1.9

In basic solution (pH $\leqslant$ 8) GA is totally in acidic form, and so the peak of GA obtained is quite broad. Thus, it is better to calculate the total GA content in acidic standards from the relationship between the peak area of D-glucaro-1,4-lactone and the internal standard.

The precision of a series of GA samples is shown in Table II.

In the standard curve the relationship between the total amount of D-glucaric acid and the peak area of D-glucaro-1,4-lactone divided by the peak area of the internal standard (glycolic acid) showed a linear response over the concentration range 0.5—3 mg/ml.

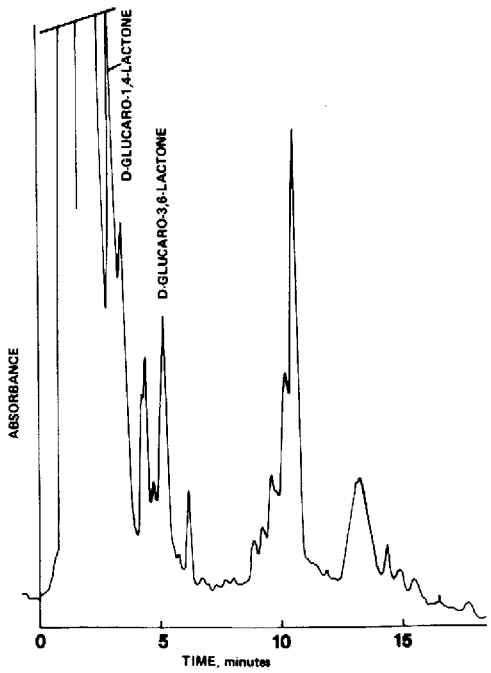


Fig. 3. HPLC chromatogram of concentrated normal human urine, when the D-glucaro-lactones are qualitatively identified by co-elution with their standard solution.

The detectable amount of GA in aqueous solution is 2—4 μg at 220 nm.

The chromatogram of a urine sample obtained from a healthy subject is shown in Fig. 2.

Fig. 3. is a chromatogram of the same urine sample, in which the D-glucaro-1,4-lactone and the 3,6-lactone have been qualitatively identified by co-elution with the standard solution of lactones.

DISCUSSION

The results show that GA and its lactones can be analyzed quantitatively in aqueous solution by HPLC on the weak anion-exchange column Spherisorb-NH_2. The column should be used in the anion-exchange mode to separate the compounds with ionizable protons. In the straight phase they presumably form salts with the amino groups in the column packing. The amino column might be preserved for longer with ammonium acetate—acetonitrile or ammonium acetate—methanol as eluant, but this was not checked. The amino column has earlier been found to be suitable for the analysis of some organic acids [23, 24]. Possibly the D-glucaro-3,6-lactone could be used for quantification of GA, too, because it separates well and has a rather low variation coefficient.

Baseline noise is quite disturbing at wavelengths as low as 220 nm. In the blank, however, it remained satisfactory. By preparing a colored derivative, the measurements could be made at a visible wavelength.

By this method GA can only be qualitatively demonstrated in urine, because the peaks of D-glucarolactones do not separate well enough to allow quantitative determination. The urine samples should first be purified. The internal standard glycolic acid stays under the matrix, and normal urine contains a little of it [25].

Certain organic acids in urine have been analyzed by HPLC [26, 27] but D-glucaric acid was not included.

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