Galactonic Acid in Galactosemia: Identification in the Urine

Abstract. *Galactose is converted to galactonic acid in vivo in man. Galactonate was isolated from the urine of galactosemia patients who had been given galactose orally. The identity of the galactonate was established by gas-liquid chromatography and by the preparation of derivatives.*

Galactonic acid has been isolated from the urine of galactosemia patients that have been given an oral dose of galactose. Lesser amounts of this metabolite were found in the urine of a normal individual studied in the same manner. This result may be interpreted as an indication of the existence of an oxidative pathway for the metabolism of galactose in man.

Cuatrecasas and Segal described a pathway in mammalian liver in which galactose is converted by a galactose dehydrogenase (D-galactose : NAD oxidoreductase; E.C. 1.1.1.48) to galactonate, which then undergoes oxidation and decarboxylation to D-xylulose (*1, 2*). The presence of a galactose dehydrogenase in mammalian tissues was disputed by Beutler, who confirmed the experimental results of Cuatrecasas and Segal but suggested that the enzyme responsible for the effects observed is alcohol dehydrogenase (alcohol : NAD oxidoreductase; E.C. 1.1.1.1) and that the substrate is alcohol, which contaminates the reagents (*3*). Evidence obtained by Shaw and Koen indicated that liver galactose dehydrogenase and alcohol dehydrogenase are the same enzyme, although contamination of their purified alcohol dehydrogenase with a separate galactose dehydrogenase was not completely excluded (*4*).

Cuatrecasas and Segal found that galactose dehydrogenase activity is primarily in the soluble cellular fraction of rat liver (*2*). Srivastava and Beutler could not demonstrate the formation of unphosphorylated galactonic acid in supernatant fraction of rat liver homogenates (*5*). However, they showed that 6-phosphogalactonic acid was formed from galactose 6-phosphate in the presence of hexose-6-phosphate dehydrogenase, a microsomal enzyme from rat liver.

We have now identified galactonic acid as a by-product of galactose metabolism in the human. Three galactosemic patients (ages 20 to 24 years) were given a single oral dose of galactose: 35 g/m^2 (60 g). Urine samples were collected during the 6-hour period just prior to administration of the dose, and then during each of the 6-hour intervals afterward, up to 24 to 48 hours. Blood samples were drawn just before the galactose was administered, and again 0.5, 1.5, 3, 8, 12, and 24 hours later. All three of the galactosemic patients have been followed by G. N. Donnell since infancy. Two are Caucasians, one male and one female, and the third is a 20-year-old Negro male. All have been under good dietary management. No subjective or objective evidence of distress was detected in any of the patients upon receiving the galactose. A 26-year-old normal Caucasian male served as a control.

In all cases, the expected response in blood galactose concentration was obtained. With the galactosemic patients, the plasma concentration of galactose rose to more than 200 mg per 100 ml of plasma and remained elevated (in excess of 30 mg/100 ml) for more than 6 hours. With the normal control, the concentration of galactose in the plasma returned to zero by 3 hours. Concentrations of galactose 1-phosphate in the erythrocytes of the galactosemic patients rose to a maximum of 30 mg per 100 ml of packed cell volume, and after 24 hours they still were two to three times the baseline values. Erythrocyte galactose 1-phosphate was determined by a modification of the method of Gitzelmann (see *6*).

Sugars in the urine were determined by a modification of the gas-liquid chromatography method of Wells (*7*), in which α-methylmannoside was used as an internal standard, and the urease treatment was omitted. Silylation was effected in pyridine with a mixture of hexamethyldisilazane and trimethylchlorosilane. The procedure demonstrates galactitol and galactonic acid as well as urine sugars. Galactonate, either as calcium salt for reference or as present in urine, undergoes silylation with greater difficulty, as compared to galactose or galactitol, but a 24-hour reaction period gave consistently reproducible results. The sample was injected in pyridine solution. The system does not differentiate between galactonate trimethylsilyl derivatives of galactonate and gluconate; hence other means of discrimination were employed.

Data obtained with one of the galactosemic patients, together with values for the normal individual, are shown in Table 1. Two separate studies, several weeks apart, were done with this patient. The second was carried out to examine the possibility that urinary galactonate might originate from intestinal fermentation, and therefore this subject was given neomycin orally for 4 days.

The differences between the normal

Table 1. Urinary excretion of galactose, galactitol, and galactonate from oral galactose tolerance tests.

Period of 6-hour collection*	Galactose or metabolite excreted								
	Normal adult			Galactosemic patient					
	No antibiotic			No antibiotic			Antibiotic		
	Sugar (g)	Alcohol (mg)	Acid (mg)	Sugar (g)	Alcohol (mg)	Acid (mg)	Sugar (g)	Alcohol (mg)	Acid (mg)
− 6 to 0	0	0	0	0	109	0	0	85	39
0 to 6	7.3	82	300	32.4	570	2200	43.2	320	1730
6 to 12	0	74	119	0.072	115	430	0.088	198	879
12 to 18	0	25	44	0	192	650	0	265	406
18 to 24	0	16	0	0	172	194	0	242	227
24 to 30				0	68	75	0	46	17
30 to 36				0	62	68	0	33	55
36 to 42				0	108	63	0	33	14

* 0 hour is the time at which the dose was given.

individual and the galactosemic patients with respect to galactitol and galactose output were consistent with our previous experience. The normal individual excreted about 10 percent of the galactose given, and the galactosemic patients excreted more than 50 percent.

The large amounts of galactonate excreted by the galactosemia patient are of special interest. For each of the three patients, the amount of galactonate excreted during the period of study was more than six times that found for the normal individual. If the urinary loss of a large proportion of the ingested galactose is excluded, the galactonate recovered represents almost one-fifth of the galactose that was retained by the patients. The study made after administration of the antibiotic tends to rule out intestinal flora as a source of the galactonate found.

Initially, identification of a particular peak on the chromatograms as galactonic acid was based on comparison with the authentic compound. Further confirmation was obtained by isolation and characterization. Galactonic acid was isolated from urine by column chromatography with Dowex-1. As a marker, 0.5 μc of [1-^{14}C]galactonate (specific activity of 1.54 μc/μmole) was added to the urine. The radioactive galactonate was prepared enzymatically from [1-^{14}C]galactose. Galactonate was eluted as a single broad peak with 0.1*M* ammonium formate. Gas-liquid chromatography of a portion of lyophilized eluate showed one major peak corresponding to the retention time of galactonate. A minor peak (less than 1 percent) corresponded to galactonolactone. Galactonate was precipitated as calcium salt in the presence of ethanol. After two recrystallizations, overall recovery of the dried product varied from 25 to 30 percent.

The galactonobenzimidazole derivative was prepared according to the method of Moore and Link (*8*). The purified derivative had a decomposition point at 244°C (at a heating rate of 3° per minute in a bath initially at 240°C). This value corresponded closely to that for the product prepared from calcium galactonate and to that reported by Moore and Link (245°C). In contrast, the product prepared from authentic potassium gluconate was found to have a decomposition point at 215°C, similar to that noted by Moore and Link.

Another means of characterization was the conversion of the isolated galactonate to the galactonolactone in 1*N* HCl in a boiling water bath for 5 minutes. Authentic calcium galactonate and potassium gluconate were treated under similar conditions. Samples with and without conversion were chromatographed on Whatman No. 1 paper in a descending system with ethyl acetate, pyridine, water (150 : 50 : 40). The lactones were detected by spraying the paper with alkaline hydroxylamine and with acidic ferric iron solutions (*9*). Gluconolactone and galactonolactone were clearly separated. The result obtained with the compound isolated from urine corresponded to that with authentic galactonolactone.

Urine samples containing galactitol and presumed galactonate were studied by Markey (*10*), using combined gas chromatography–mass spectrometry [Beckman GC-45 gas chromatograph coupled to an MS-12 mass spectrometer (AEI, Manchester, England) by means of a fritted separator]. A glass column (6 feet by ⅛ inch, inside diameter) containing 5 percent OV-22 on 80- to 100-mesh Chromosorb-W was used to separate the silylated urine filtrate. Scans were continuously recorded every 10 seconds on a PDP81/D computer (DEC, Maynard, Mass.) and were automatically converted to *m/e* versus intensity files (*11*). Mass spectra recorded during the first major peak were identical to those reported for the trimethylsilyl derivative of galactitol (*12*). Mass spectra recorded during the second major peak were identical to those obtained from authentic trimethylsilyl derivatives of galactonate. However, absolute structure assignment for the compound of the second peak was not possible because nearly identical fragmentation patterns and retention values were obtained with authentic trimethylsilyl derivatives of gluconate (*12, 13*).

Final confirmation of the presence of galactonic acid was obtained by another gas-liquid chromatographic system. The retention times and mass spectra of the methanolyzed, acetylated urine filtrate were compared with those from a standard solution of sorbitol and galactitol acetates and the methyl esters of galactonic and gluconic acetate. The compounds were chromatographically separated isothermally (165°C) in a column containing 3 percent ECNSS-M on 80- to 100-mesh Chromosorb W (*14*).

The possibility that the urinary galactonate may have been derived from contamination of the galactose that was fed was excluded. No detectable amount of galactonate could be demonstrated in an amount of galactose equivalent to the dose. The possible origin of galactonate by nonenzymatic or by bacterial action in the urine itself also was excluded. Urine with added galactose was stored for varying periods of time (up to 10 days) under conditions simulating those for the handling of patient samples. No formation of galactonate was found.

The consistent finding of urinary galactonate after an oral administration of a single large dose of galactose, both in galactosemic patients and in a normal individual, indicated the presence in vivo of an oxidative pathway for galactose. The finding of the unphosphorylated product is consistent with the galactonate pathway proposed by Cuatrecasas and Segal, but the conditions of the study do not exclude a phosphorylated galactose intermediate.

It is still not known whether infants and children have the same capability as the adults studied; the overall significance of an oxidative pathway in relation to galactosemia remains to be determined.

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