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Galactonate determination in urine by stable isotope dilution gas chromatography–mass spectrometry

Peter Schadewaldt*, Hans-Werner Hammen, Sara Stolpmann, Loganathan Kamalanathan, Udo Wendel

Klinik für Allgemeine Pädiatrie, Universitätsklinikum, Heinrich-Heine-Universität Düsseldorf, Metabolic Unit, Moorenstraße 5, D-40225 Düsseldorf, Germany

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Abstract

A stable isotope dilution assay was developed for the sensitive determination of D-galactonic acid. D-[U-$^{13}C_6$]galactono-1,4-lactone was prepared as internal standard. Unlabelled and U-^{13}C-labelled D-galactonic acid species were converted to the *N*-(1-butyl)galactonamide pentaacetate derivatives and assessed by gas chromatography–mass spectrometry (GC–MS). Positive chemical ionisation and monitoring of the $[M\text{H-}60]^+$-ions in the galactonate chromatographic peak at *m*/*z* 402 and *m*/*z* 408 were used for quantification. The procedure was applied to study the variability of D-galactonate excretion in healthy subjects and galactosemic patients and to monitor the D-galactonate–D-galactitol ratio in human urine.
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Keyword: Galactonate

1. Introduction

In humans, D-galactose from the diet and from endogenous sources is normally disposed via the consecutive action of galactokinase (EC 2.7.1.6), galactose-1-phosphate uridyltransferase (EC 2.7.7.12) and UDP-galactose-4-epimerase (EC 5.1.3.2), and thus channelled into glucose metabolism. Inherited deficiencies of the enzyme activities of this pathway cause galactosaemia, the most frequent being classical galactosaemia, i.e., galactose-1-phosphate uridyltransferase deficiency (OMIM 230400, [1]). Principally, D-galactitol and D-galactonic acid formation and excretion may provide alternative pathways for D-galactose disposal (see [2] for review).

About 30 years ago, Bergren et al. demonstrated D-galactonic acid formation in humans by showing the appearance of D-galactonic acid in urine after oral flooding with high amounts of D-galactose [3]. Since then, only a few studies addressed this alternative of D-galactose metabolism in humans [4,5]. For example, the questions whether the primary fate of D-galactonic acid once formed in the body is renal excretion rather than further degradation via the xylulose pathway [3,6] and of the physiological significance of D-galactonic acid excretion in galactosaemia are still unanswered [2].

For D-galactonic acid analysis, gas chromatography, sometimes combined with mass spectrometric detection, of the trimethylsilyl derivative is the method most commonly applied [3,7–14]. Use of the pentaacetyl aldonmethylester [15] or derivatization with butylboroacetate [10] have also been proposed. A procedure specifically converting aldonic acids into the *N*-alkylamide peracetylated derivatives has been described by Lehrfeld [16,17]. Furthermore, ion exchange chromatography [18], ion exclusion chromatography [19,20], HPLC separation of the phenylisocyanate derivative [21], capillary (zone) electrophoresis [22–24], and recently ^{1}H and ^{13}C NMR [4,5] have been suggested for D-galactonic acid quantification.

None of the published methods, however, is of sufficient sensitivity to allow measurements in normal human body fluids and some fail to separate D-galactonic from D-gluconic acid derivatives [3,10,18]. In addition, with the exception of the procedure described by Lehrfeld, the methods are complicated by the tendency of aldonic acids to form

* Corresponding author. On leave from the Deutsches Diabetes Forschungsinstitut. Tel.: +49-211-81-16965; fax: +49-211-81-16969.
E-mail address: schadewa@uni-duesseldorf.de (P. Schadewaldt).

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lactones [16,17]. The relative amounts of lactone to free acid may vary with pH, temperature, time and solvent. Thus total D-galactonic acid concentration may be underestimated when only one species is monitored [8,16,17].

In the present communication we modified the method of Lehrfeld [16] and developed a sensitive and reliable stable isotope dilution assay for gas chromatographic–mass spectrometric assessment of total D-galactonic acid in human urine. D-[U-^{13}C]galactono-1,4-lactone was prepared as internal standard and selected ion monitoring of the [*M*H-60]$^{+}$-ions of the unlabelled and ^{13}C-labelled *N*-[1-butyl]galactonamide pentacetate derivatives was used for quantification.

2. Materials and methods

2.1. Chemicals and enzymes

Unless otherwise noted, all chemicals were obtained in the highest available purity from Merck, Darmstadt, Germany, or Fluka Sigma–Aldrich, Seelze, Germany. Ion exchange resins were from Serva, Heidelberg, Germany. Alkaline phosphatase, E.C. 3.1.3.1, from calf intestine, and β-D-galactose dehydrogenase, EC 1.1.1.48, from *Pseudomonas fluorescens*, and coenzymes were supplied by Roche, Mannheim, Germany. D-[U-^{14}C]galactose-1-phosphate (11 Gbq/mmol) was obtained from Biotrend, Cologne, Germany, D-[U-^{13}C]galactose (98.5% U-$^{13}C_6$; 1.5% unlabelled) was obtained from Promochem, Wesel, Germany. Stable isotope labelled galactitol and galactonate were commercially unavailable. Therefore, D-[U-^{13}C]galactitol and D-[U-^{13}C]galactono-γ-lactone were prepared as standards from D-[U-^{13}C]galactose essentially as described in [25] and [26], respectively. For preparation of D-[U-^{13}C]galactitol, D-[U-^{13}C]galactose (10 mg) was reacted with $NaBH_4$ (20 mg dissolved in diluted NaOH, 50 mmol/l, 1 ml) at ambient temperature overnight. The solution was neutralized by addition of acetic acid (2 mol/l) and purified by Dowex 1X8 and Dowex 50 WX8 ion exchange chromatography as described below. D-[U-^{13}C]galactonate was prepared as γ-lactone from D-[U-^{13}C]galactose as follows: D-[U-^{13}C]galactose (10 mg) was dissolved in Tris–HCl (1.8 ml, 0.25 mol/l, pH 8.7) fortified with NAD^+ (70 mg) and galactose dehydrogenase (1.5 U, 60 μl). After incubation at ambient temperature for 10 h, HCl (6 mol/l, 0.15 ml) was added and the solution stored at ambient temperature overnight to allow γ-lacton formation to go to completion. Finally, the D-[U-13C]galactono-1,4-lactone preparation was purified by ion exchange chromatography as described below. Concentrations of the labelled standards were determined by inverse isotope dilution gas chromatography–mass spectrometry (GC–MS) (cf. below) using unlabelled preparations of pure D-galactitol and D-galactono-1,4-lactone (from Sigma–Aldrich, Munich, Germany). The final yields were 80 and 46% for D-[U-^{13}C]galactitol and D-[U-^{13}C]galactono-1,4-lactone, respectively. As checked by gas chromatography–mass spectrometry (see below), the D-[U-^{13}C]galactitol and the D-[U-^{13}C]galactono-1,4-lactone preparation were pure (98.5% U-$^{13}C_6$) and contained <0.2% unreacted D-[U-^{13}C]galactose.

2.2. Subjects

The relation of D-galactose metabolites in human urine samples was studied in patients with galactose-1-phosphate uridyltransferase deficiency [11 females and 10 males; age (mean ± S.D.), 16 ± 10 (range 4–39) years; weight, 40 ± 21 (13–77) kg; height, 147 ± 28 (98–189) cm], obligate heterozygous parents (12 women and 12 men; age, 42 ± 8 years; weight, 74 ± 17 kg; height, 172 ± 10 cm), and healthy adults (11 women and 9 men; age, 29 ± 8 years; weight, 67±15 kg; height, 174±9 cm). After an overnight fast >10 h, samples of spontaneous urine were collected between 07:00 and 09:00 a.m., chilled on dry ice and then stored at −20 °C until analysis.

D-Galactose-1-phosphate uridyltransferase activity in red blood cells was determined according to [27] and galactose-1-phosphate according to [26]. Creatinine was measured enzymatically [28] on a Hitachi 912 analyser (from Boehringer).

The study had been approved by the Ethikkommitte of the Heinrich-Heine-Universität Düsseldorf, and written informed consent was obtained from all subjects participating in the study.

2.3. Sample pre-treatment and extraction

In general, urine specimens from galactosemic patients (diluted 1:5) and the other subjects (0.1 ml each) were spiked with D-[U-^{13}C]galactitol and D-[U-^{13}C]galactono-1,4-lactone (5 nmol each), adjusted to a total volume of 0.27 ml, mixed with HCl (6 mol/l, 0.03 ml), and stored overnight at ambient temperature to allow D-galactono-1,4-lactone formation to go to completion [16]. H_2O was then added to give a final volume of 2 ml. For purification the sample was applied onto a Dowex 1X8 column (200–400 mesh, acetate-form; 4 ml in disposable Poly Prep chromatography columns from Bio-Rad, Munich, Germany) and allowed to elute. The eluate was discarded. D-Galactose metabolites were eluted from the column using H_2O (2 ml). The latter eluate was applied onto a Dowex 50 WX8 column (200–400 mesh, H^+-form; 4 ml in disposable columns, cf. above) followed by a wash with H_2O (2 ml). The final 2 ml of the eluate were collected, transferred into a reaction vial and evaporated to dryness under a stream of gaseous N_2 [29].

For analysis of D-galactono-1,4-lactone and D-galactitol the *N*-(1-butyl)galactonamide pentaacetate and galactitol hexaacetate derivative, respectively, were prepared essentially as described by Lehrfeld [16]. Butylamine in pyridine (1:1 (v/v), 0.1 ml) was added to the dry extracts and the

solutions maintained at 90 °C for 30 min. After evaporation to dryness as above, acetic acid anhydride in pyridine (1:1 (v/v), 0.1 ml) was added and the solutions maintained at 90 °C for 60 min. After evaporation, the residues were extracted with hexane (0.1 ml) and evaporated as above. The final residue was dissolved in ethyl acetate (50 μl) and then subjected to analysis by gas chromatography–mass spectrometry as described below.

2.4. GC–MS procedure

A HP 6890 gas chromatograph equipped with a DB-5 MS capillary column (5% phenylmethylpolysiloxane; 30 m, i.d. 0.25 mm, 0.25 μm film thickness; J&W Scientific, Folrom, CA) and directly connected to a HP mass-selective detector (HP MSD) was used (Hewlett-Packard (now Agilent), Waldbronn, Germany). Helium was the carrier gas ($0.9\,ml\,min^{-1}$). One to two microlitres of sample was injected in splitless mode. The injector and the transfer line to the spectrometer were held at 250 °C. The column temperature was 125 °C initially. After 1.5 min, the temperature was increased to 190 °C and further to 260 °C at ramp rates of 20 and 5 °C/min, respectively, and then raised to 280 °C for 2 min. Positive chemical ionisation was applied, using methane as a reactant gas. The ion source was operated at 170 °C. Source pressure was 60 mPa.

$^{12}C_6$- and U-$^{13}C_6$-labelled metabolite species were assessed by selected ion monitoring of the $[M\text{H-60}]^+$-ion intensities at *m/z* 375 and *m/z* 381, respectively, in the galactitol chromatographic peak, and at *m/z* 402 and *m/z* 408, respectively, in the galactonate chromatographic peak.

2.5. Calculations

The concentrations (*c*, in μmol/l) of naturally occurring D-galactitol and D-galactonate were calculated from the ratios (*R*) of the ion intensities (*m/z* 375)/(*m/z* 381) and (*m/z* 402)/(*m/z* 408), respectively, using the equation

$$c = \{(RF_C A_{St} P_{St}) - [(1 - P_{St})A_{St}]\}\frac{1}{V_U} \quad (1)$$

where F_C (1.07) is a factor correcting for the higher base peak intensities of the U-^{13}C-labelled species in the respective $[M\text{H-60}]^+$-ion clusters, A_{St} the added (nominal) amount of ^{13}C-labelled internal standard (in μmol), P_{St} the proportion of U-^{13}C-labelled molecules in the standard (0.985; $1 - P_{St}$ accounts for the proportion of unlabelled metabolite added to the sample together with the standard), and V_U the sample volume of urine (in l).

2.6. Statistics

Unless otherwise noted, results are presented as means ± S.D. with the number of separate determinations in parentheses. When assessed by D-galactitol measurements in urine samples from a healthy subject and a galactosemic patient, within-run ($n = 8$) and between-run ($n = 10$) CVs at lower concentrations (∼30 μmol/l) were 6.1 and 7.3%, respectively, and at higher concentrations (∼1360 μmol/l) 2.1 and 2.4%, respectively. Linearity was checked using spiked urine samples from a healthy subject. D-Galactitol analysis was linear in the low (spike 0–200 μmol/l, eight concentrations, linear regression: $y = 1.02(\pm0.01)x + 8.3(\pm1.4)$, $s_{y/x} = 2.3$, $R^2 > 0.999$, with *y* and *x* being the found and added concentrations) and in the high concentration range (spike 0–4.0 mmol/l, eight concentrations, linear regression: $y = 0.97(\pm0.01)x + 0.02(\pm0.02)$, $s_{y/x} = 0.03$, $R^2 > 0.999$). The overall analytical CV for creatinine related urinary D-galactitol ($CV_{total} = \sqrt{CV^2_{galol} + CV^2_{crea}}$) in healthy subjects and patients was 7.5 and 3.1%, respectively [30]. Correlations were checked by linear regression analysis (least squares method; using the RGP function of MS Excel). For examination of differences, the Mann–Whitney *U*-test was used.

3. Results

3.1. CI fragmentation pattern

The fragmentation pattern of *N*-(1-butyl)galactonamide pentaacetate as obtained in the positive chemical ionisation mode with methane as reactant gas is shown in Fig. 1. Characteristic ions retaining all six carbons of naturally occurring D-galactonic acid were found at the following *m/z* values: 490, 462, 420, 402, 360, 342, 300, 284, and 242 (Fig. 1A). Likely, the ions at *m/z* 402 and 342 were due to the loss of acetic acid moieties ($C_2H_4O_2$, −60 u) from the pseudo molecular ion $[M\text{H}]^+$ at *m/z* 462, and the fragments at *m/z* 420, 360, and 300 were due to the additional loss of a keten moiety (C_2H_2O, −42 u) from the former species. Similarly, the ions at *m/z* 242 were probably formed by keten loss from the fragments at *m/z* 284. The formation of the latter species is not as readily explained. Possibly, it is derived from the $[M\text{H-120}]^+$-ion by consecutive losses of CH_2O and CO (−58 u). The ions appearing at *m/z* 490 were most likely formed by addition of ethylene moieties (C_2H_4, +28 u) to the pseudo molecular ions. With U-$^{13}C_6$-labelled galactonic acid, the fragmentation pattern was practically identical with the fragments appearing at 6 mass units higher *m/z* values (Fig. 1B).

The ion abundance was largely concentrated (about 80%) on the $[M\text{H}]^+$- and $[M\text{H-60}]^+$-ion species at *m/z* 462 and *m/z* 402, respectively, with the derivative of the unlabelled species, and, consequently, at *m/z* 468 and *m/z* 408, respectively, with the derivative of the U-^{13}C-labelled species. The ratio of $[M\text{H}]^+/[M\text{H-60}]^+$ was 0.7. Efforts to concentrate the ion abundance on one of these species remained unsuccessful.

Therefore, selected ion monitoring at *m/z* 402 and *m/z* 408 was used for quantification. Representative chromatograms

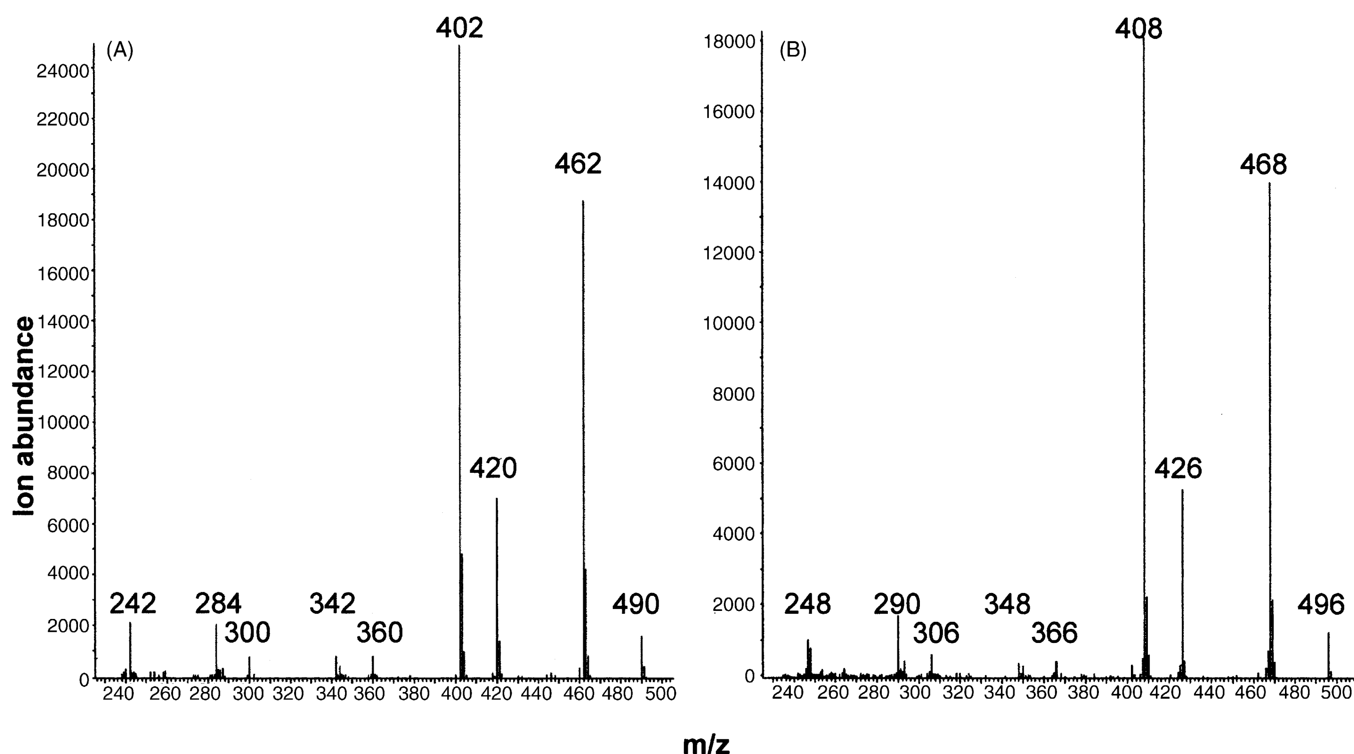

Fig. 1. Fragmentation pattern of the *N*-(1-butyl)-pentaacetate derivatives of unlabelled (A) and U-$^{13}C_6$-labelled D-galactonic acid (B) using positive chemical ionisation with methane as reactant gas. The pseudo molecular $[MH]^+$-ion and the $[MH\text{-}60]^+$-ion are the most prominent components appearing at *m/z* 462 and *m/z* 402, respectively, for the unlabelled species and at *m/z* 468 and *m/z* 408, respectively, for the U-^{13}C-labelled compound.

as obtained with urine samples from galactosemic patients and healthy subjects are shown in Fig. 2. Noteworthy, the presence of D-gluconic acid in the samples did not interfere, because the gluconamide derivative eluted well separated about 0.5 min prior to the galactonamide derivative.

3.2. Completeness, linearity and precision

Besides D-galactonic acid, varying amounts of the derived 1,4-lactone may also be present in physiological fluids. Therefore, we first examined completeness of lactone formation under our assay conditions. A diluted urine sample was fortified with Ca-D-galactonate and D-galactono-1,4-lactone at variable relations and analysed for total D-galactonic acid content. The data compiled in Table 1 clearly show that the results were not affected by the initial D-galactonate–D-galactono-1,4-lactone ratio. This indicated that both galactonic acid species are quantified equally reliable by the present procedure.

Linearity of the gas chromatographic–mass spectrometric procedure was checked using urine specimens from a galactosemic patient and a healthy subject after spiking with authentic D-galactonate in amounts to cover the concentration range relevant for classical galactosaemia (0.1–2 mmol/l) and for healthy subjects (0.01–0.2 mmol/l), respectively. Excellent linear correlations were obtained between added and measured concentration in both cases (see Fig. 2). Under the present assay conditions, the detection limit as evaluated in analyses of 10 normal urine specimens implying a galactonate peak signal to noise ratio of 3:1 was 1.0 ± 0.4 μmol/l (Fig. 3).

Imprecision was examined using unspiked urine samples from a healthy subject (D-galactonate concentration, ∼40 μmol/l) and from a galactosemic patient under dietary control (D-galactonate concentration, ∼161 μmol/l). Within-run ($n = 10$) and between-run ($n = 10$) CVs at the lower concentration were 1.5 and 7.1%, respectively, and at the higher concentration 1.5 and 8.4%, respectively.

Table 1
Assessment of total galactonate content in human urine by GC–MS analysis of the *N*-[1-butyl]galactonamide pentaacetate derivative using D-[U-^{13}C]galactono-1,4-lactone as labelled standard

Spike of galactonic acid species[a]		Total galactonate concentration[b]
D-Galactonate (Ca salt)	D-Galactono-1,4-lactone (μmol/l)	
100	0	98 ± 2
80	20	99 ± 1
60	40	99 ± 2
40	60	99 ± 1
20	80	101 ± 1
0	100	98 ± 3

[a] Urine from a postabsorptive healthy subject was enriched with the naturally labelled species to give the nominal concentrations indicated.
[b] Results are means ± S.D. from four determinations.

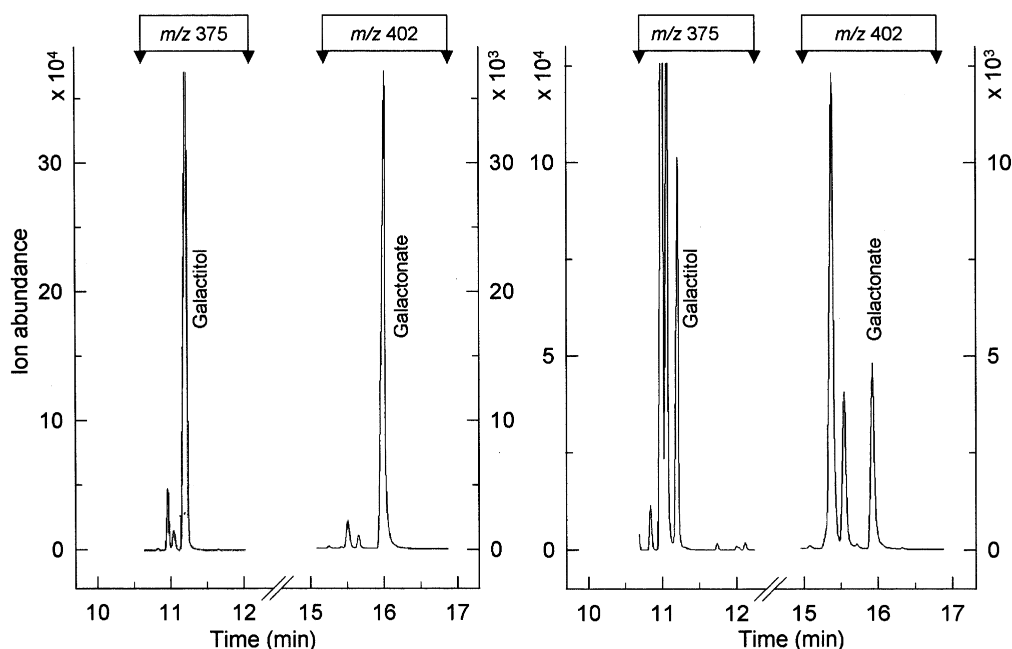

Fig. 2. Gas chromatographic-mass spectrometric analysis of D-galactonate and D-galactitol in human urine. Spot urine specimens from a postabsorptive patient with galactose-1-phosphate uridyltransferase deficiency (A) and from a healthy subject (B) were worked up as detailed in Section 2. Methane positive chemical ionisation and selected ion monitoring of the $[M\text{H-}60]^+$-ion intensities as indicated were used for detection of the *N*-(1-butyl)galactonamide pentaactate and the galactitol hexaacetate derivatives. *Note*: As assessed by stable isotope dilution analysis, sample concentration of D-galactonate in (A) and (B) was 175 and 37 μmol/l, respectively, the concentration of D-galactitol in (A) and (B) was 809 and 40 μmol/l, respectively.

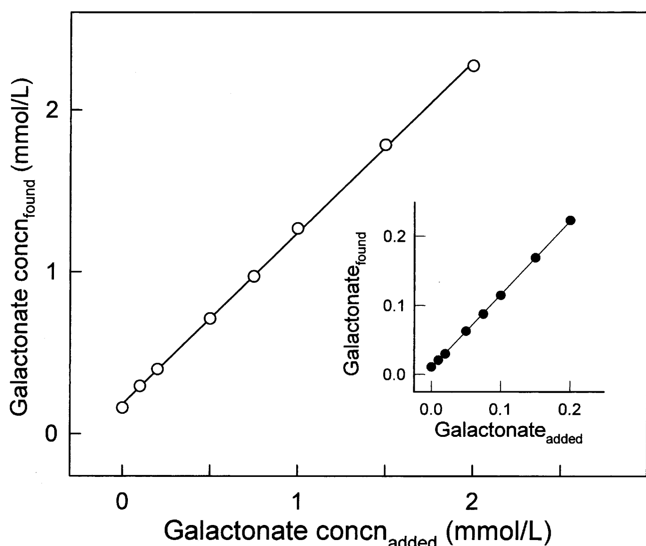

Fig. 3. Linearity of the D-[U-^{13}C]galactono-1,4-lactone based stable isotope dilution assay for assessment of D-galactonate in human urine. Increasing amounts of naturally labelled D-galactonate (Ca salt) were added to spot urine samples from a patient with classical galactosaemia (open circles) and a healthy subject (inset, filled circles) as indicated; samples were then analysed for galactonate as detailed in Section 2. Regression lines (linear regression, least squares method): open circles: $y = 1.06(\pm0.01)x + 0.18(\pm0.01)$; $s_{y/x} = 0.03$, $R^2 > 0.996$; inset, filled circles: $y = 1.06(\pm0.01)x + 0.010(\pm0.001)$; $s_{y/x} = 0.001$, $R^2 > 0.999$. Intercepts represent estimates of D-galactonate concentrations in the original urine specimens.

3.3. Relation of D-galactose metabolites in urine

The assay was used to examine the relation of D-galactonate and D-galactitol excretion in galactosemic patients under dietary treatment, obligate heterozygous parents and healthy adults. The data are compiled in Table 2. Postabsorptive galactosemic patients excreted considerable and variable amounts of D-galactonate (concentration range 79–949 μmol/l, equivalent to 24–66 $\mu mol/mmol_{creatinine}$) and D-galactitol (238–4831 μmol/l, equivalent to 100–306 $\mu mol/mmol_{creatinine}$). However, the ratio of D-galactonate to D-galactitol excretion was rather constant at about 1:4.

We also monitored the intraindividual variability of postabsorptive metabolite excretion. The course over a 4.5-week period in a patient with classical galactosaemia is shown in Fig. 4. When corrected for the total analytical imprecision of urinary D-galactonate and D-galactitol analysis, the intraindividual CVs were <10%. Confirmatory observations have been made in five other patients.

In urine of the obligate heterozygous parents, D-galactonate excretion (6–99 μmol/l, equivalent to 0.4–8.6 $\mu m\,mol/mmol_{creatinine}$) and D-galactitol excretion (17–94 μmol/l, equivalent to 1.3–3.8 $\mu mol/mmol_{creatinine}$) was substantially lower than in galactosemic patients, as expected. Galactonate and galactitol excretion amounted to only about 6 and 1%, respectively, when related to the mean urinary metabolite disposal in the patients. The ratio of D-galactonate and D-galactitol was highly variable with a mean of 1.2 ± 0.7. Quite comparable findings were made in the healthy adults under study (Table 2).

Table 2
Relation of galactonate and galactitol in urine of postabsorptive subjects[a]

Subjects	Galactonate (μmol/mmol$_{creatinine}$)	Galactitol (μmol/mmol$_{creatinine}$)	Ratio
Galactosemic	42 ± 13	185 ± 62	0.23 ± 0.03
Patients (n = 21)[b]	(39; 24–66)	(157; 100–306)	(0.22; 0.18–0.33)
Heterozygous	2.8 ± 2.1	2.2 ± 0.7	1.2 ± 0.7
Parents (n = 24)[c]	(2.3; 0.4–8.6)	(2.2; 1.3–3.8)	(1.1; 0.2–2.5)
Healthy	2.5 ± 2.7	1.6 ± 0.4	1.3 ± 1.0
Adults (n = 20)[d]	(1.7; 0.4–11.6)	(1.7; 0.6–2.1)	(1.1; 0.1–4.6)

[a] Overnight fast, postabsorptive period >10 h; results are means ± S.D. with median and range in parentheses.
[b] Patients with galactose-1-phosphate uridyltransferase activity (GALT) in erythrocytes (RBC) <1% of control under dietary treatment (mean galactose-1-phosphate concentrations, 132 ± 33 μmol/l$_{RBC}$).
[c] GALT activity, 50 ± 5% of control.
[d] GALT activity, 22 ± 3 μmol/(h g) Hb.

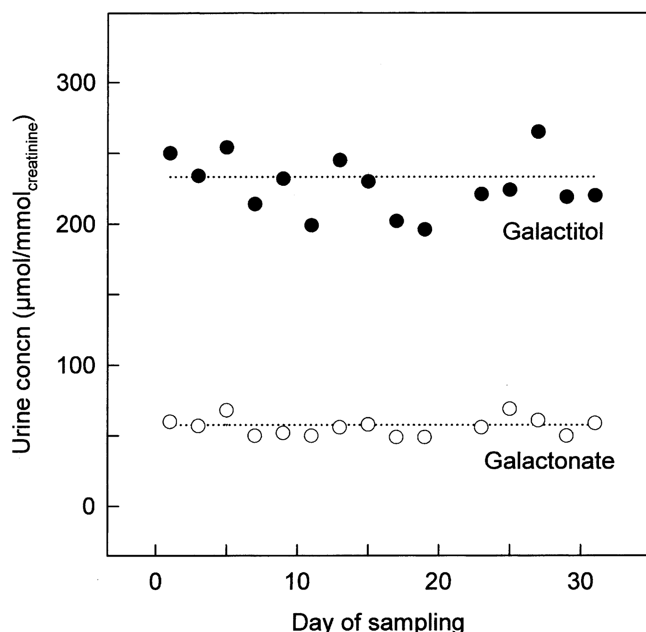

Fig. 4. Intraindividual variability of D-galactonate and D-galactitol excretion into urine in a patient with classical galactosaemia. Morning urine specimens were collected in a patient with galactose-1-phosphate uridyltransferase deficiency over a period of about 4 weeks as indicated. Concentrations of D-galactonate and D-galactitol were assessed by stable isotope dilution analysis as detailed in Section 2 and related to creatinine content. Means are indicated by dashed lines.

4. Discussion

The present procedure was designed to allow a reliable and sensitive measurement of D-galactonate together with D-galactitol in human urine specimens. As D-galactose excretion is known to be comparatively low, excretion of D-galactonate and D-galactitol can provide a pathway for whole body D-galactose disposal [2]. This may be of physiological importance in the different forms of galactosaemia, where the common Leloir pathway of D-galactose disposal is impaired by inherited enzyme deficiencies [4,30].

The principles of stable isotope dilution analysis of D-galactitol in human body fluids have been established by Jakobs et al. some time ago [25] and the procedure has been applied extensively on a previous occasion [30]. For a discussion of D-galactitol analysis by gas chromatography–mass spectrometry we therefore refer to that previous work and concentrate on D-galactonate analysis in the present communication.

Stable isotope dilution analysis by mass spectrometric methods is recognized as a powerful tool for metabolite quantification, especially when used in conjunction with selected ion monitoring. For D-galactonate analysis, an U-^{13}C-labelled standard was prepared in order to minimize interferences from the isotopic cluster of the naturally occurring compound. All major components in the CI mass spectrum retained the carbons of D-galactonate. Taken together that may be of advantage especially for label incorporation studies applying specifically labelled D-galactose precursors, e.g. D-[1-^{13}C]galactose, the substrate of choice for in vivo D-galactose turnover studies [29].

For gas chromatographic–mass spectrometric analysis, we chose the *N*-(1-alkyl)aldonamide peracetylated derivative. The present derivatization procedure appears to have several advantages over the commonly applied derivatization with *N*,*O*-bistrimethylsilyltrifluoroacetamide. (i) The D-galactonamide derivative is comparatively stable. (ii) It is easily separated from the D-gluconamide derivative. (iii) In positive chemical ionisation mode, ion intensity was concentrated on two high molecular weight species, i.e. the pseudo molecular ion and the [MH-60]$^+$-ion, allowing sensitive selected ion monitoring. (iv) The procedure was quantitative and yielded one single compound, irrespective of the relation of acid and lactone in the original sample. The latter is of special importance because the relation may be variable in physiological samples. Furthermore, the relation may change rather rapidly during sample workup, because lactone formation is very sensitive to alterations of pH and temperature [16,17].

In fact, the present procedure appears to be at least one order of magnitude more sensitive than all previously reported methods. It allowed for the first time the detection and quantification of D-galactonate in urine specimens from healthy subjects. In all previous studies, irrespective of the principles

used for analysis, D-galactonate in normal urine was undetected unless subjects were loaded with rather generous amounts of D-galactitol (50–60 g) [3,4]. The present results in postabsorptive subjects now suggest that D-galactonate formation is a general, although minor, pathway of human D-galactose disposition.

Even in galactosaemia, the quantitative aspects and the significance of D-galactonate formation are not well understood at present. A few fragmentary data on urine concentrations have been obtained by gas chromatography–mass spectrometry [3], one more extended study used ^{1}H and ^{13}C NMR analysis [4]. In the latter study, D-galactonate in random urine samples from treated galactosemic patients was in the range 20–100 $\mu mol/mmol_{creatinine}$ and the relation of D-galactitol to D-galactonate was about 3:1. These data are rather comparable to the present findings in urine specimens of postabsorptive galactosemic patients. With respect to the intraindividual variability of D-galactose metabolite excretion, however, no data appear to have been reported in the literature. In any case, the present method, due to its sensitivity and the suitability for label enrichment analysis, makes now feasible future more detailed experimental approaches on D-galactonate formation in normal subjects and patients, including stable isotope in vivo turnover studies.

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