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Analysis of Concentration and ^{13}C Enrichment of D-Galactose in Human Plasma

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Background: **A stable-isotope dilution method for the sensitive determination of D-galactose in human plasma was established.**

Methods: **D-[^{13}C]Galactose was added to plasma, and the concentration was measured after D-glucose was removed from the plasma by treatment with D-glucose oxidase and the sample was purified by ion-exchange chromatography. For gas chromatographic–mass spectrometric analysis, aldononitrile pentaacetate derivatives were prepared. Monitoring of the $[MH\text{-}60]^+$ ion intensities at *m/z* 328, 329, and 334 in the positive chemical ionization mode allowed the assessment of 1-^{12}C-, 1-^{13}C-, and U-$^{13}C_6$-labeled D-galactose, respectively. The D-galactose concentration was quantified on the basis of the ^{13}C-labeled internal standard.**

Results: **The method was linear (range examined, 0.1–5 μmol/L) and of good repeatability in the low and high concentration ranges (within- and between-run CVs <15%). The limit of quantification for plasma D-galactose was <0.02 μmol/L. Measurements in plasma of postabsorptive subjects yielded D-galactose concentrations (mean ± SD) of 0.12 ± 0.03 (n = 16), 0.11 ± 0.04 (n = 15), 1.44 ± 0.54 (n = 10), and 0.17 ± 0.07 (n = 5) μmol/L in healthy adults, diabetic patients, patients with classical galactosemia, and obligate heterozygous parents thereof, respectively. These data were considerably lower (3- to 18-fold) than the values of a conventional enzymatic assay. The procedure was also applied successfully in a stable-isotope turnover study to evaluate endogenous D-galactose formation.**

Conclusions: **The present findings establish that detection of D-galactose from endogenous sources is feasible in human plasma and show that erroneously high results may be obtained by enzymatic methods.**

In patients with classical galactosemia [McKusick 2304000, *(1)*], the catabolism of D-galactose is severely impaired because of an inherited D-galactose-1-phosphate uridyltransferase (EC 2.7.7.10) deficiency. In galactosemic infants on an unrestricted lactose intake, a potentially lethal organ toxicity syndrome develops, presumably because D-galactose-derived metabolites (D-galactose-1-phosphate and D-galactitol) accumulate within the cells. Patients improve rapidly on cessation of lactose intake. A strict and lifelong dietary restriction of D-galactose is the recommended form of therapy. Even when patients are on an extremely D-galactose-restricted diet, however, long-term disturbances emerge, e.g., retarded development in intellectual performance, possible neurologic symptoms, and hypergonadotropic hypogonadism in most females [see Segal and Berry *(2)* for a comprehensive review]. Gitzelmann and Steinmann *(3)* supposed that these long-term complications might be attributable to production of free D-galactose from endogenous sources, leading to an "autointoxication" in galactosemic patients. In fact, isotope kinetic tracer experiments performed in a limited number of adult patients and healthy subjects indicated that substantial and comparable amounts of D-galactose are produced in both study groups *(4)*.

Knowledge of the quantitative role of endogenous production of D-galactose is essential for judgment of the significance of lifelong dietary restrictions in patients with hereditary galactosemia. Therefore, it seemed important to examine a possible age dependency of endogenous galactose production rates in galactosemic patients. For this purpose, in vivo measurement of D-galactose turnover in postabsorptive subjects by use of ^{13}C-labeled D-galactose infusion represents the method of choice *(4)*. When we were planning an appropriate study, we found

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that numerous enzymatic, gas chromatography (GC),[3] and HPLC procedures had been published, but no method has been detailed in the literature that is sufficiently sensitive to allow reliable estimation of the ^{13}C enrichment or concentration of plasma D-galactose in postabsorptive subjects and patients. Henderson et al. *(5)* applied an enzymatic fluorometric method and originally reported on fasting concentrations in human plasma in the range of ~5–100 μmol/L. Enzymatic measurements in plasma are known to be subject to interferences, however, and there are indications that D-galactose may be overestimated by these procedures, especially when analyses are performed at low plasma concentrations *(5–8)*. Using HPLC and electrochemical detection, Watanabe and Kawasaki *(6)* established the presumably most sensitive assay (detection limit, 2.2 μmol/L) for D-galactose in human plasma communicated to date. These authors were actually unable to detect free D-galactose in postabsorptive subjects and stated, "Absence of free galactose in human plasma is usually the case unless deliberately added or infused" *(6)*.

Here we report on a stable-isotope dilution method for the sensitive and reliable measurement of D-galactose in plasma by use of 1-^{13}C- or U-$^{13}C_6$-labeled D-galactose. The major obstacle in the determination of D-galactose by GC-mass spectrometry (MS) procedures, i.e., the presence of comparatively large amounts of D-glucose in plasma samples, has been overcome by the use of an enzymatic D-glucose removal step. The method allowed for the first time estimation of free plasma D-galactose in postabsorptive subjects. The usefulness of the procedure for evaluation of ^{13}C label enrichment in plasma D-galactose is demonstrated by measurements in samples from a stable-isotope study on D-galactose turnover performed in a healthy subject.

Materials and Methods

SUBJECTS AND PATIENTS

For the determination of fasting D-galactose concentrations, venous EDTA blood samples were collected between 0700 and 0900 from healthy adults [4 women and 12 men; age, 33 ± 8 years (mean ± SD); weight, 72 ± 6 kg; height, 180 ± 8 cm], patients with diabetes mellitus (8 women and 8 men; age, 56 ± 12 years; weight, 84 ± 14 kg; height, 167 ± 9 cm), patients with classical galactosemia [6 females and 4 males; age, 17 ± 10 years; weight, 44 ± 19 kg; height, 147 ± 25 cm; galactose-1-phosphate uridyltransferase activity in erythrocytes measured according to Shin *(9)*, <2% of control], and obligate heterozygous parents of the galactosemic patients (2 women and 3 men; age, 43 ± 16 years; weight, 78 ± 29 kg; height; 174 ± 10 cm; galactose-1-phosphate uridyltransferase, 49% ± 5% of control). All subjects were studied in the postabsorptive state after an overnight fast of ≥10 h. Plasma was separated by centrifugation (3000*g* for 10 min at 4 °C).

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

GALACTOSE TURNOVER STUDY

A primed continuous infusion test was performed under resting conditions essentially as described by Berry et al. *(4)*. In short, after an overnight fast, a healthy volunteer (male; age, 25 years) received an intravenous priming dose of D-[1-^{13}C]galactose (8 μmol/kg of body weight) at ~0900. Thereafter, a continuous infusion of 0.8 μmol D-[1-^{13}C]galactose · kg body weight^{-1} · h^{-1} via a cannula inserted into the basilic vein was started and continued for 6 h. D-Glucose was infused intravenously at a rate of 11 μmol · kg body weight^{-1} · min^{-1} from ~0800 until the end of the experiment. Samples of venous EDTA blood were collected from the cannula placed on the contralateral arm, and plasma was prepared for analysis as described above.

CHEMICALS AND ENZYMES

Unless otherwise noted, all chemicals were obtained in the highest available purity from Merck or Sigma Chemie. Ion-exchange resins were from Serva. Catalase (EC 1.11.1.6, from bovine liver), D-galactose dehydrogenase (EC 1.1.1.48, from *Pseudomonas fluorescens*), D-glucose oxidase (EC 1.1.3.4, from *Aspergillus niger*), D-glucose-6-phosphate dehydrogenase (EC 1.1.1.49, from yeast), hexokinase (EC 2.7.1.1, from yeast), and coenzymes were purchased from Boehringer Mannheim.

D-[1-^{13}C]Galactose (99% 1-^{13}C, according to the manufacturer) and D-[U-$^{13}C_6$]galactose (99% U-$^{13}C_6$, according to the manufacturer), produced by Cambridge Isotope Laboratories, were obtained from Promochem. According to our GC-MS analysis (see below), however, the ^{13}C label enrichment in the D-[1-^{13}C]galactose preparation was 97.0% ± 0.1% (n = 14; duplicates analyzed on 7 different working days). The purity of the uniformly labeled D-galactose was 98.5% ± 0.2% (n = 6 on 2 different working days).

ENZYMATIC GALACTOSE AND GLUCOSE ASSAYS

A modification of the procedure described by Fujimura *(10)* was used: D-Galactose solutions containing 0 (basal value), 10, or 50 μmol/L (25 μL each) were added to three plasma aliquots (0.2 mL). After the plasma was deproteinized by the addition of perchloric acid (0.1 mL of a 1.5 mol/L solution) and centrifugation (13 000*g* for 10 min at 4 °C), the supernatant (0.25 mL) was neutralized with 50 μL of 2.5 mol/L $KHCO_3$, and the $KClO_4$ was then removed by centrifugation (see above). The neutralized extract (0.25 mL) was mixed with 0.6 mL of 1 mol/L Tris-HCl buffer (pH 8.7) and 0.25 mL of 5 mmol/L NAD^+. After fluorescent blanks were measured (λ_{ex} = 340 nm;

[3] Nonstandard abbreviations: GC, gas chromatography; MS, mass spectrometry; and MPE, mole-percentage of enrichment.

λ_{em} = 450 nm; Model 650 fluorometer; Perkin-Elmer), the reaction was started by the addition of 10 μL of D-galactose dehydrogenase solution (0.25 U). After ~60 min, a stable end-point fluorescent signal was reached, indicating that the enzymatic reaction had gone to completion. The D-galactose concentration in the sample to which 0 μmol/L D-galactose had been added was then calculated on the basis of the increase in fluorescent intensities of the two samples to which 0.25 or 1.25 μmol/L D-galactose had been added. In the recovery studies (see *Results*) with plasma (pools) to which D-galactose had been added, appropriate blanks were prepared with authentic plasma samples containing no added D-galactose.

D-Glucose concentrations were measured spectrophotometrically using the hexokinase-D-glucose-6-phosphate dehydrogenase assay essentially as described by Kunst et al. *(11)*.

PLASMA EXTRACTION AND GLUCOSE REMOVAL

For internal standardization, 20 μL of a 50 μmol/L D-[1-^{13}C]galactose solution was added to 1 mL of plasma. D-[U-$^{13}C_6$]Galactose solution (20 μL of a 50 μmol/L solution) was added to plasma samples from the in vivo galactose turnover study. Water was then added to give a final volume of 1.25 mL, and the sample was mixed with 0.25 mL of 3 mol/L perchloric acid for deproteinization. After centrifugation (13 000*g* for 5 min at 4 °C), 1.2 mL of the supernatant was mixed with 0.2 mL of 2.5 mol/L $KHCO_3$ and 0.1 mL of 2.5 mol/L potassium phosphate buffer (pH 6.5). The $KClO_4$ was removed by centrifugation (see above). For enzymatic removal of D-glucose, catalase (65 kU in 50 μL) and D-glucose oxidase (10–15 U in 50 μL) were added to 1.3 mL of buffered extract. The mixture was equilibrated with air and incubated at 25 °C for 90 min. The reaction was stopped by the addition of 0.15 mL of 4.5 mol/L perchloric acid and centrifuged (see above). The supernatant (1.4 mL) was mixed with $KHCO_3$ (0.25 mL of a 2.5 mol/L solution), the $KClO_4$ removed by centrifugation, and the glucose-depleted extract was purified by subsequent ion-exchange chromatography.

SAMPLE PURIFICATION AND DERIVATIZATION

A 1.5-mL aliquot of the above extract was applied onto a Dowex 1 × 8 column (200–400 mesh, acetate form; 4 mL in disposable PolyPrep chromatography columns from Bio-Rad); 0.25 mL of water was then applied to the column and allowed to elute. The eluate was discarded. D-Galactose was eluted from the column with 2 mL of H_2O. The latter eluate was applied onto a Dowex 50 WX8 column (200–400 mesh, H^+ form; 4 mL in disposable columns; see above) followed by a wash with 2 mL of H_2O. The final 2 mL of the eluate was collected, transferred into a reaction vial, and evaporated to dryness under a stream of gaseous N_2.

For preparation of aldononitrile pentaacetates, the dry residue was reacted with 50 μL of hydroxylamine hydrochloride (0.3 mol/L in pyridine) at 90 °C for 30 min. Thereafter, 50 μL of acetic anhydride was added, and the reaction mixture was kept at 90 °C for 60 min. After evaporation under a stream of gaseous N_2, the dry residue was extracted with 0.1 mL of hexane. The hexane was evaporated as above. The final residue was dissolved in 50 μL of ethyl acetate and then subjected to galactose analysis by GC-MS as described below.

GC-MS PROCEDURE

A HP 6890 gas chromatograph equipped with a HP-5 MS capillary column [5% phenylmethylpolysiloxane; 30 m × 0.25 mm (i.d.); 0.25 μm film thickness] and directly connected to a HP mass selective detector was used (Hewlett-Packard). Helium was the carrier gas (0.9 mL/min). Sample (1.0 μL) was injected in the splitless mode. The injector and the transfer line to the spectrometer were held at 250 °C. The initial column temperature was 150 °C. After 0.5 min, the temperature was increased to 250 °C at a ramp rate of 10 °C/min and then raised to 280 °C for 2 min. Positive chemical ionization was used with methane as the reactant gas. The ion source was operated at 170 °C. Source pressure was 60 mPa. Selected ion monitoring of the $[MH\text{-}60]^+$ ion intensities at *m/z* 328, 329, and 334 in the galactose chromatographic peak allowed the assessment of 1-^{12}C-, 1-^{13}C-, and U-$^{13}C_6$-labeled D-galactose, respectively.

CALCULATIONS

To assess the natural ^{13}C enrichment in plasma hexoses, we measured the ion intensities at *m/z* 328 and 329 in the D-galactose and D-glucose chromatographic peaks in a representative number of native plasma samples. The ratio R_0 = (*m/z* 329)/(*m/z* 328) was 0.159 ± 0.003 (mean ± SD; n = 20) for either hexose, and this value was used for further calculations.

We used the ion intensity ratio, R_1 = (*m/z* 329)/(*m/z* 328), in the galactose chromatographic peak to estimate the concentration of D-galactose (C_{gal}, in μmol/L) in the plasma samples to which D-[1-^{13}C]galactose (97% 1-^{13}C, 3% naturally labeled; final concentration, 1 μmol/L) had been added (taking into account the amount of naturally labeled D-galactose in the D-[1-^{13}C]galactose preparation) according to the equation:

$$C_{gal} = 0.97 \times \frac{1}{R_1 - R_0} - 0.03 \qquad (1)$$

In plasma samples collected in the primed continuous infusion experiment, to which D-[U-$^{13}C_6$]galactose preparation (98.5% U-$^{13}C_6$, 1.5% naturally labeled; final concentration, 1 μmol/L) had been added, the ion intensity ratios R_{U1} = (*m/z* 334)/(*m/z* 328) and R_{U2} = (*m/z* 334)/(*m/z* 329) in the D-galactose chromatographic peak were used to estimate the concentration of total D-galactose (C_{tot}, i.e., sum of naturally labeled and D-[1-^{13}C]galactose, in μmol/L) according to the equation:

$$C_{tot} = 0.985 \times \left[(1 - R_0) \frac{1}{R_{U1}} + \frac{1}{R_{U2}} \right] - 0.015 \quad (2)$$

The amount of naturally labeled D-galactose (C_{nat}, μmol/L) in the sample was estimated according to the equation:

$$C_{nat} = 0.985 \times \frac{1}{R_{U1}} - 0.015 \quad (3)$$

and the amount of 1-^{13}C-labeled D-galactose in the sample (C_{13C}, μmol/L) was estimated using the equation:

$$C_{13C} = 0.985 \times \left(\frac{1}{R_{U2}} - R_0 \times \frac{1}{R_{U1}} \right) \quad (4)$$

The amount of infused exogenous D-galactose (naturally labeled plus 1-^{13}C-labeled; C_{exo}, μmol/L) is given by:

$$C_{exo} = \frac{1}{0.97} C_{13C} \quad (5)$$

Thus, the concentration of D-galactose attributable to endogenous sources (C_{endo}, μmol/L) in the primed continuous infusion experiment can be estimated applying the equation

$$C_{endo} = C_{tot} - C_{exo} \quad (6)$$

The rate of appearance of endogenous D-galactose in plasma under apparent steady-state conditions (R_a, in μmol · kg body weight^{-1} · min^{-1}) was calculated from the ratio of ^{13}C-labeled to total D-galactose and the infusion rate (*Inf*, in μmol · kg body weight^{-1} · min^{-1}) and corrected for the amount of natural enriched D-galactose in the infusate as follows:

$$R_a = Inf \times \left(1 - \frac{C_{13C}}{C_{tot}} \right) - 0.03 \times Inf \quad (7)$$

The mole-percentage of enrichment of 1-^{13}C label in plasma D-galactose (*MPE*) was calculated according to the equation:

$$MPE = \frac{C_{13C}}{C_{tot}} \times 100 \quad (8)$$

STATISTICS

In general, results are presented as the mean ± SD with the number of separate determinations in parentheses. Correlations were checked by linear regression analysis (least-squares method). For examination of differences, the Mann–Whitney *U*-test was used.

Results

EFFICIENCY OF GLUCOSE REMOVAL

D-Glucose oxidase-catalyzed conversion of D-glucose in plasma extracts was essentially complete at the end of the 90-min incubation period (Fig. 1). As tested with two plasma pools containing 7.0 ± 0.1 and 18.2 ± 0.5 mmol/L D-glucose (n = 7) to which 10 μmol/L D-galactose had been added, the concentration of D-glucose at the end of incubation was reduced to 0.020 ± 0.002 and to 0.058 ± 0.019 mmol/L, respectively. The concentration of D-galactose remained unaffected and was 10.1 ± 0.5 and 9.8 ± 0.2 μmol/L, respectively (n = 7). The efficiency of D-glucose removal was 99.77% ± 0.03% and 99.71% ± 0.08% (n = 7), respectively. Thus, the excess of D-glucose over D-galactose in the pools was reduced by more than 300-fold, from ~700:1 and 1800:1 to 2:1 and 6:1, respectively.

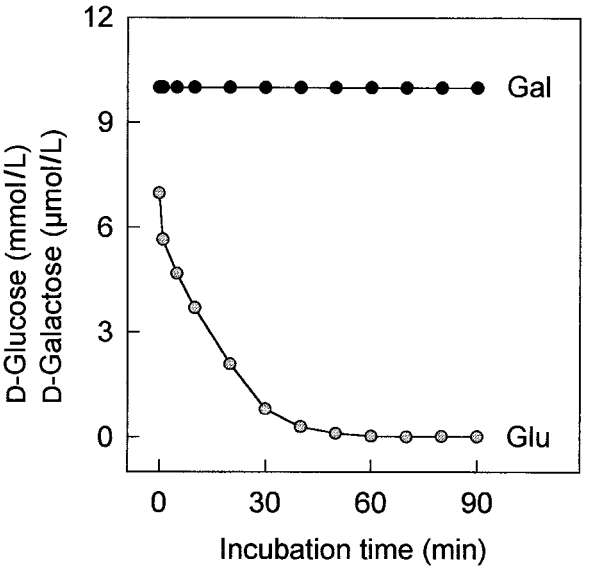

Fig. 1. Time course and specificity of D-glucose removal from plasma samples by use of D-glucose oxidase.

Human plasma containing 7 mmol/L D-glucose (○) was enriched with D-galactose (●; 10 μmol/L) and deproteinized buffered extract (pH 6.5) treated at 25 °C with D-glucose oxidase from *A. niger* (10 kU/L) in the presence of catalase (50 MU/L). At the time periods indicated, aliquots were withdrawn from the mixture, and the D-glucose and D-galactose concentrations were measured (see *Materials and Methods* for details).

The recovery of D-galactose in the purification procedure was checked using 1-mL plasma samples with 5 μmol D-galactose/L added. A total of 2.3 ± 0.2 μmol D-galactose was recovered after the ion-exchange chromatographic sample clean-up (n = 11). This is equivalent to 81% ± 7% of the maximal theoretical yield (2.8 μmol), which can be estimated on the basis of the inevitable losses. Some D-galactose had obviously been lost in either chromatographic step.

The efficacy of the procedure is demonstrated in Fig. 2, which shows typical GC-MS chromatograms as obtained in analyses of authentic human plasma. In postabsorptive healthy subjects, the ratio of D-glucose to D-galactose in plasma was ~30 000–60 000:1 (see below). Without D-glucose oxidase treatment, D-galactose eluted as a tiny rear rider or shoulder peak of a huge D-glucose chromatographic peak. Therefore, reliable measurement of D-galactose was impossible. In contrast, in the D-glucose-depleted

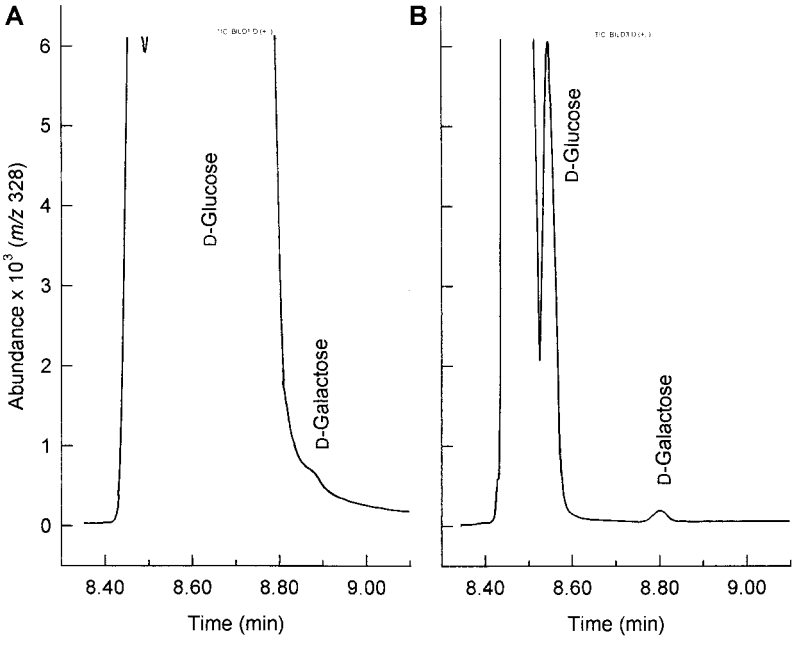

Fig. 2. GC-MS chromatograms showing the effect of enzymatic removal of D-glucose on the separation of the aldononitrile pentaacetate derivatives of D-glucose and D-galactose from human plasma.

Aliquots of plasma from a postabsorptive healthy subject were worked up in parallel as detailed in *Materials and Methods* but without the addition of D-[1-^{13}C]galactose. Sample preparations for *A* and *B* differed in that glucose oxidase was absent in the workup of the sample in *A*. Positive chemical ionization was used for detection. Traces for the $[MH\text{-}60]^+$ ion intensities (*m/z* 328) are shown.

samples, good separation of the two peaks was achieved, thus allowing quantification of the D-galactose chromatographic peak.

LINEARITY AND PRECISION

Preliminary measurements in human plasma by the D-galactose dehydrogenase assay suggested postabsorptive D-galactose concentrations of ~1 μmol/L. Therefore, 1 nmol of D-[1-^{13}C]galactose was added for each milliliter of sample in the stable-isotope dilution assay. Before performing GC-MS determinations in authentic plasma samples, we examined the linearity and repeatability of this approach. The results are summarized in Fig. 3 and Table 1, respectively.

In matrix-free as well as in plasma samples, we obtained excellent linear correlation of the *m/z* 329/328 ratio and the ratio of D-[1-^{13}C]-labeled to naturally labeled galactose (r >0.9999; see Fig. 3). When imprecision was examined using human plasma pools containing low (~0.2 μmol/L) and increased (~1.3 μmol/L) concentrations of natural D-galactose, the within- and between-run CVs were ≤5% and <15%, respectively (Table 1).

PLASMA GALACTOSE IN POSTABSORPTIVE SUBJECTS

The stable-isotope dilution assay was then applied to the assessment of free D-galactose in human plasma. Postabsorptive subjects were investigated to avoid uncontrollable and variable contributions of exogenous D-galactose from the diet. The results are shown in Table 2. In healthy subjects (n = 16), diabetic patients (n = 15), and obligate heterozygous parents of patients with the classical form of galactosemia (n = 5), plasma concentrations of D-galactose were similarly low, with mean values of ~0.1 μmol/L. Postabsorptive patients with classical galactosemia (n = 10), however, exhibited ~10-fold increased ($P \ll 0.001$) plasma D-galactose concentrations.

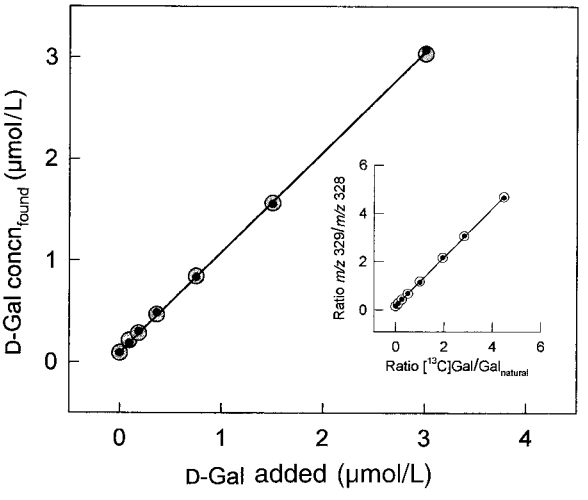

Fig. 3. Linearity of the D-[1-^{13}C]galactose stable-isotope dilution assay for measurement of D-galactose concentrations.

Increasing amounts of naturally labeled D-galactose were added to plasma from a healthy subject as indicated; the plasma was then analyzed for total D-galactose concentrations as detailed in *Materials and Methods*. Results from two series of analyses are shown (● and *larger shaded circles*). Regression line (linear regression analysis, least-squares method): y = 0.979 (± 0.004)x + 0.104 (± 0.005); $S_{y|x}$ = 0.014; r >0.9999; n = 14. The intercept represents the estimate of the D-galactose concentration in the original plasma sample. (*Inset*), matrix-free solutions of naturally labeled and 1-^{13}C-labeled D-galactose were mixed to give the ratios of ^{13}C-labeled and naturally labeled material indicated. Samples were then analyzed by GC-MS for the ensuing ratio of the $[MH\text{-}60]^+$ ion intensities of the aldononitrile pentaacetate derivatives at *m/z* 329 and 328. Results from two series of analyses are shown (● and *larger open circles*). Regression line (linear regression analysis, least-squares method): y = 1.015 (± 0.004)x + 0.166 (± 0.007); $S_{y|x}$ = 0.021; r >0.9999; n = 16. The intercept represents the estimate of the ratio *m/z* 329/328 in the naturally labeled D-galactose.

Table 1. Repeatability of methods for the assessment of D-galactose in human plasma.

	CV,[b] %			
	Stable-isotope dilution assay		Enzymatic assay	
Plasma pool[a]	Within-run (n = 10)	Between-run (n = 10)	Within-run (n = 10)	Between-run (n = 12)
Low concentration	5.0 (0.18 ± 0.01)	14 (0.18 ± 0.03)	3.2 (2.4 ± 0.1)	7.2 (1.6 ± 0.1)
High concentration	3.4 (1.32 ± 0.04)	2.4 (1.28 ± 0.03)	2.1 (5.1 ± 0.1)	6.0 (6.6 ± 0.4)
	Limit of quantification,[c] μmol/L			
	<0.02		<0.2	

[a] Different human plasma pools were prepared, and pure D-galactose was added in various concentrations. D-Glucose concentrations were 7–11 mmol/L.
[b] Apparent D-galactose concentrations (mean ± SD) as measured with the respective methods are in parentheses (see Fig. 3).
[c] In human plasma, applying the procedures detailed in *Materials and Methods*.

COMPARISON OF METHODS

Somewhat unexpected was the observation that our preliminary measurements by the D-galactose dehydrogenase assay (see above) indicated considerably higher concentrations of plasma D-galactose in postabsorptive subjects than were measured in the D-[1-^{13}C]galactose stable-isotope dilution assay. This prompted us to check the enzymatic assay by performing a series of comparative measurements. Data on the precision of the enzymatic assay are included in Table 2. The results obtained in the individual samples are shown in Fig. 4, and a summary of the data is given in Table 3. In fact, when compared with the data of the stable-isotope dilution assay, plasma concentrations of D-galactose in healthy adults and in diabetic patients appeared to be overestimated ~10-fold by the enzymatic assay. In galactosemic patients exhibiting higher plasma concentrations of D-galactose, the mean apparent overestimation was still ~3-fold. Interestingly, the additional amount detected by the enzymatic assay [nongalactosemic subjects, +0.91 ± 0.50 μmol/L (median, 0.75; range, 0.26–2.21 μmol/L; n = 30); galactosemic subjects, +2.54 ± 1.42 μmol/L (median, 1.94; range, 0.58–5.19 μmol/L; n = 9)] was rather variable, and no statistically significant correlation between the data of the GC-MS and the enzymatic method was observed in any of the three study groups.

Table 2. Concentration of free D-galactose in plasma of postabsorptive subjects.

Subjects[a]	D-Galactose plasma concentration,[b] μmol/L	n
Healthy adults	0.12 ± 0.03 (0.11; 0.08–0.18)	16[c]
Diabetic patients[d]	0.11 ± 0.04 (0.11; 0.07–0.21)	15[c]
Galactosemic patients[e]	1.44 ± 0.54[e] (1.59; 0.44–2.27)	10
Obligate heterozygous parents[f]	0.17 ± 0.07 (0.20; 0.08–0.26)	5

[a] Overnight fast, postabsorptive period ≥10 h.
[b] As assessed by the present D-[1-^{13}C]galactose stable-isotope dilution assay. Median and range given in parentheses.
[c] Samples were analyzed twice; deviation of individual values from the mean values was 3% ± 3% (0–10%) in healthy subjects and 7% ± 5% (0–16%) in diabetic patients.
[d] Blood D-glucose was 9.9 ± 2.8 mmol/L (range, 5.8–15.1 mmol/L).
[e] Galactose-1-phosphate uridyltransferase deficiency; statistically significantly different from the other study groups ($P << 0.001$).
[f] Parents of the galactosemic patients.

GALACTOSE TURNOVER STUDY

We examined the applicability of the present GC-MS procedure for the evaluation of stable-isotope turnover studies. The results of a primed continuous infusion

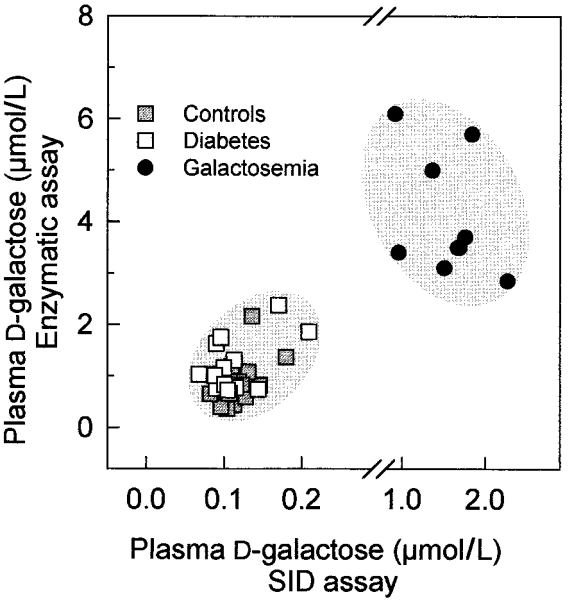

Fig. 4. Comparison of plasma concentrations of D-galactose in postabsorptive healthy subjects and patients as measured by the D-[1-^{13}C]galactose isotope dilution (*SID*) assay and the enzymatic D-galactose dehydrogenase assay.

See *Materials and Methods* for details. The results obtained by the two methods appeared to be statistically uncorrelated (r <0.5, linear regression, least-squares method). For convenience, *shaded areas* indicating high and low concentration ranges are under the data from nongalactosemic and galactosemic subjects.

Table 3. Plasma D-galactose in postabsorptive subjects as assessed by the D-galactose dehydrogenase assay.

Subjects[a]	Apparent D-galactose concentration in plasma,[b] μmol/L	Apparent overestimation,[b,c] fold	n[a]
Healthy adults	0.9 ± 0.5	7.6 ± 3.5	16
	(0.8; 0.4–2.2)	(7.5; 3.5–15.9)	
Diabetic patients	1.2 ± 0.5	10.8 ± 4.3	14
	(1.0; 0.7–2.4)	(10.2; 5.1–18.2)	
Galactosemic patients	4.1 ± 1.2[d]	3.0 ± 1.6[d]	9
	(3.5; 2.9–6.1)	(2.1; 1.3–6.7)	

[a] Same subjects as in Table 2; shortage of plasma volume in one diabetic and one galactosemic patient.

[b] Mean ± SD; median and range given in parentheses.

[c] When compared with the data obtained by the present D-[1-^{13}C]galactose stable-isotope dilution assay (see Fig. 3).

[d] Statistically significantly different from the other study groups ($P < 0.001$).

experiment with D-[1-^{13}C]galactose as performed in a healthy volunteer are shown in Fig. 5. Within ~3 h of D-[1-^{13}C]galactose infusion, a satisfactory stable steady state of ^{13}C enrichment in plasma D-galactose (76 ± 2 MPE; n = 7) was reached. According to these data, the estimated rate of appearance of endogenous D-galactose in plasma was 0.17 μmol · kg body weight^{-1} · h^{-1} in this adult subject. With D-[U-^{13}C$_6$]galactose for internal standardization, it could also be shown that the portion of the plasma concentration derived from endogenous sources

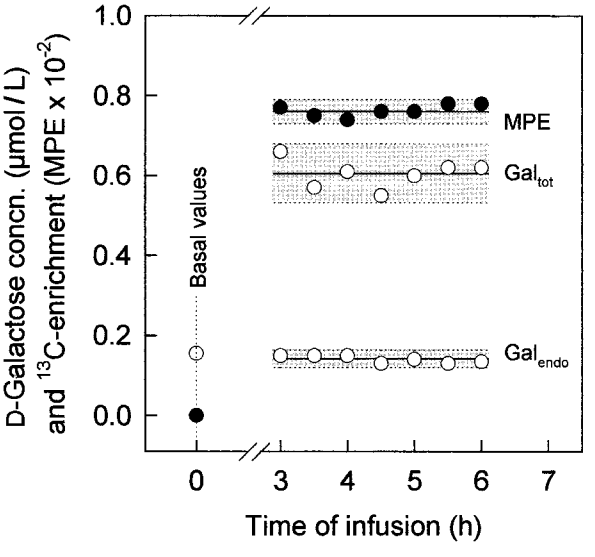

Fig. 5. D-Galactose concentration and 1-^{13}C enrichment (*MPE*) in plasma of a postabsorptive healthy subject undergoing a 6-h primed continuous infusion test with D-[1-^{13}C]galactose.

The priming and infusion doses were 8 μmol/kg of body weight and 0.8 μmol · kg body weight^{-1} · h^{-1}, respectively. Control samples (*basal values*) were collected before application of the priming dose. 1-^{13}C enrichment and plasma total D-galactose (*Gal$_{tot}$*) were analyzed using GC-MS procedures. For concentration measurements, D-[U-^{13}C$_6$]galactose was used as internal standard. The portion of endogenous D-galactose in a sample (*Gal$_{endo}$*) was estimated on the basis of MPE and the total concentration of D-galactose. See *Materials and Methods* for details. ●, ^{13}C enrichment; ○, D-galactose concentration. *Lines* and *shaded areas* indicate means and ± 2 SD, respectively.

remained essentially constant during the course of the experiment (Fig. 5).

Discussion

The presence of comparatively large amounts of D-glucose is the major problem for reliable measurement of low D-galactose concentrations in human plasma by GC-MS methods. Our present data indicate that, under postabsorptive conditions, the ratios of excess D-glucose over D-galactose in the plasma of healthy adults, diabetic patients, and patients with classical galactosemia are 30 000–60 000:1, 50 000–200 000:1, and 2 000–10 000:1, respectively. The present results show that interferences from D-glucose can be readily overcome by effective and specific removal of this compound from plasma samples by the use of D-glucose oxidase from *A. niger (12, 13)*. The data further show that only minor losses of D-galactose occur during the subsequent ion-exchange purification procedure, provided that sample clean-up is careful.

To date, the method for plasma D-galactose with the lowest detection limit (2.2 μmol/L) appears to be the HPLC-electrochemical detection method described by Watanabe and Kawasaki *(6)*. Thus, the detection limit of the present procedure is more than two orders of magnitude lower than in previously described D-galactose assays *(5–8)*.

For GC-MS analysis, we chose the aldononitrile pentaacetate derivative. This derivative has several advantages. Acetylated aldononitriles of the hexoses represent single chemical entities, whereas other derivatization procedures may yield two or more products, e.g., alkylsilylation of aldohexoses yields four cyclic tautomers in various amounts. These are separated on the GC column, thus reducing the overall sensitivity of MS detection *(14, 15)*. Furthermore, the aldononitrile pentaacetate derivatives are rather stable, and only minor fragmentation occurs using methane chemical ionization *(16)*. For the derivative of D-galactose, most of the ion current was concentrated in the [MH-60]$^+$ ion cluster, and interfering peaks were absent at *m/z* 328, 329, and 334, thus enhancing the specificity and sensitivity of quantification.

The present data now firmly establish that D-galactose is generally present in plasma of postabsorptive subjects although at very low concentrations. The concentrations measured in the in vivo study also show that D-galactose was released at a rather constant rate from endogenous sources into the plasma compartment. If the plasma D-galactose had been a remainder from dietary sources, the concentration would be expected to decline during the course of the experiment because of the comparatively high metabolic clearance of plasma D-galactose in the investigated subject (~1 μmol · kg body weight^{-1} · h^{-1}). It should be noted in this context that constancy of the isotope dilution of infused D-[1-^{13}C]galactose is only an apparent indicator of stable plasma concentrations unless gradual accumulation of naturally labeled (endogenous) D-galactose is excluded.

In our healthy subject, the rate of appearance of endogenous D-galactose in plasma (0.17 μmol · kg body weight^{-1} · h^{-1}) was remarkably low when compared with the rates observed by Berry et al. *(4)* in three healthy adults under quite comparable experimental conditions (2.9–5.4 μmol · kg body weight^{-1} · h^{-1}). In a recently conducted control experiment with the same ^{13}C dosage as has been applied by Berry et al. *(4)*, we experienced a similarly low rate of D-galactose production (0.43 μmol · kg body weight^{-1} · h^{-1}) in a second healthy adult. The causes for this apparent discrepancy are not obvious at present, and whether this points to a considerable intraindividual variation of endogenous D-galactose production remains to be elucidated.

As expected, the postabsorptive plasma concentrations of D-galactose were significantly higher in patients with the classical form of galactosemia than in nongalactosemic subjects. Whether this is attributable solely to the reduced rate of D-galactose clearance in the liver of the galactosemic patients or is also caused by a somewhat enhanced release of D-galactose from extrahepatic tissues remains to be clarified.

A conventional fluorometric D-galactose dehydrogenase assay *(10)* was modified to allow determination of D-galactose in the low concentration range. The comparative GC-MS and enzymatic measurements, however, point to the presence of (unidentified) interfering substances in human plasma that lead to erroneously high D-galactose estimates in the D-galactose dehydrogenase assay. Similar observations have been reported previously *(6–8)*. Although the enzymatic assay works perfectly well with pure solutions of D-galactose, it cannot be recommended for analysis of plasma D-galactose in the low micromolar concentration range.

In conclusion, the present GC-MS procedure is applicable for the sensitive and reliable determination of the concentration and ^{13}C enrichment of D-galactose in human plasma. It is necessary, however, to check carefully any ^{13}C-labeled D-galactose preparation before using it for internal standardization or infusion because different preparations may contain different amounts of interfering compounds *(16)*.

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