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Continuous-Flow Fluorometry of Low Galactose Concentrations in Blood or Plasma

J. Michael Henderson and Frank W. Fales[1]

Clearance of 0–100 mg/L concentrations of galactose from the blood depends on nutrient hepatic blood flow. We can measure such concentrations, which was not previously possible, by a continuous-flow method involving the use of galactose oxidase and peroxidase, the latter being coupled to a fluorogenic substrate, *p*-hydroxyphenylacetic acid. Interfering substances in the peroxidase reaction are removed by zinc/alkali precipitation. Sensitivity is maximized by using saturating concentrations of the enzymes and substrate. In prepared plasma test samples with galactose concentrations of 10, 40, 70, and 100 mg/L, the within-run CV's ranged from 2.1 to 8.6%, and day-to-day CV's from 2.2 to 17.2%, the largest CV's being for the 10 mg/L concentration. Normal subjects are shown to clear galactose more efficiently than subjects with moderate cirrhosis.

Additional Keyphrases: *hepatic blood flow · cirrhosis · liver disease assessment*

Galactose, first used to assess liver function of human subjects in 1906 (*1*), has retained its popularity with several generations of hepatic physiologists (*2–4*) because 90–95% of a test dose is metabolized in the liver (*5*). The remainder is excreted in the urine or metabolized by erythrocytes (*5, 6*). Hepatic metabolism rapidly converts it to glucose by way of galactokinase (EC 2.7.1.6), galactose-1-phosphate uridylyltransferase (EC 2.7.7.10), and UDPgalactose 4-epimerase (EC 5.1.3.2) (*7*), but the rate at which it is metabolized is a function of its concentration in plasma. In the high-concentration range (0.5 to 2 g/L), after a large intravenous bolus injection (500 mg/kg body wt.), clearance follows zero-order kinetics—i.e., the plasma concentration declines linearly with time (*8*)—and the rate-limiting factor is galactose metabolism in the hepatocyte. In contrast, in the low-concentration range (<200 mg/L), clearance exhibits first-order kinetics—the concentration in plasma declines exponentially with time—and the rate-limiting factor is the rate of blood flow through the liver (*4, 9*). Measurement of galactose clearance in this range of concentration in the blood was proposed (*4, 5*) as a method of measuring hepatic blood flow in the 1950's, but was not implemented, probably because there was no method of accurately measuring galactose in low (0–100 mg/L) concentration in the blood or plasma.

The applicability of clearance methods for measuring hepatic blood flow has been limited in the past by the inability of the cirrhotic liver to extract the test compound completely during one passage through the organ. For example, if a diseased liver can extract only 5 mg of a test substance per unit of time, the extraction will be 5% when the input is 100 mg per unit of time; but if the input is decreased to 5 mg or less per unit of time, the extraction becomes 100%. If the test substance with 100% extraction is removed from blood only by the liver, then its rate of clearance from the blood is equivalent to total nutrient blood flow. By reducing the concentration range in which galactose clearance is measured, we can approximate these ideal conditions, and thus facilitate the noninvasive estimation of hepatic blood flow. This motivated us to try to measure low concentrations of galactose in blood.

The earliest assays of blood galactose simply measured nonglucose-reducing substances (*10, 11*). Since the 1960's, however, standard assays have used the specific oxidative enzymes, galactose oxidase (EC 1.1.3.9) or galactose dehydrogenase (EC 1.1.1.48), coupled to colorimetric reactions, which in 10 duplicate determinations reportedly detected galactose concentrations as low as 80 mg/L with a standard error of 3.4 mg/L (*12, 13*). The superior sensitivity of fluorometry was exploited in galactose analysis in 1973 (*14*), and by 1977 galactosemia could be detected by examining a drop of blood with a galactose oxidase fluorometric method (*15*): high (200–600 mg/L) concentrations of galactose could be measured in small (25-μL) volumes of blood. Attempting to apply this method to our requirement of measuring <100 mg/L concentrations of galactose in larger blood samples, we found it inadequate for the needs described above; we therefore developed the new method described here.

Materials and Methods

Collection of Blood Samples

Collect 3 mL of venous blood in sodium fluoride (7.5 mg)/potassium oxalate (6 mg) anticoagulant (Vacutainer Tube M3273PS; Becton Dickinson Co., Rutherford, NJ 07070). Centrifuge for 10 min at 4 °C and separate the plasma. Plasma samples can be stored at −20 °C for up to six weeks without loss of galactose.

Alternatively, analysis can be satisfactorily performed on fresh whole blood, collected similarly. Whole-blood galactose clearance can then be calculated, which is an advantage when the assay is used to estimate hepatic blood flow.

Pre-Treatment of Samples

Deproteinize all samples by the Somogyi method (*16*), which coprecipitates uric acid or, if whole blood is used, glutathione; these compete with *p*-hydroxyphenylacetic acid as electron donors in the oxidation/reduction reaction catalyzed by peroxidase. Sample dialysis alone, which separates the galactose from the plasma proteins, does not permit assay of the galactose in the desired concentration range; it is only when the competing electron donors are removed that the fluorescent compound forms in stoichiometric amounts. The working standards are treated by the same method. This results in a 10-fold dilution of both standards and samples, and hence the actual range of galactose assayed on the analyzer is 0–10 mg/L. Samples that contain >100 mg of galactose per liter are diluted with the wash solution for repeat analysis.

Reagents

Solutions 2, 5, 6, and 11 are kept on ice throughout each analytical run. The quantities given below are sufficient for 5 h of analysis, to assay 60 samples in duplicate.

Departments of Surgery and Biochemistry,[1] and the Clinical Research Facility, Emory University School of Medicine, Emory University Hospital, 1364 Clifton Road, NE, Atlanta, GA 30322.

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1. *Stock Tris buffer, 0.1 mol/L.* Dissolve 12.1 g of tris(hydroxymethyl)methylamine (Tris), "Sigma 7-9" (Sigma Chemical Co., St. Louis, MO 63178) in 1 L of demineralized water. Adjust the pH to 7.0 with 1 mol/L HCl. This solution is stable at 5 °C for six weeks.

2. *Working Tris buffer, 10 mmol/L.* Dilute this stock solution 10-fold with demineralized water. Add 0.25 mL of Brij 35 wetting agent (30 g/L solution) per 500 mL.

3. *Stock p-hydroxyphenylacetic acid.* Dissolve 500 mg of *p*-hydroxyphenylacetic acid (Sigma) in 100 mL of demineralized water. This solution is stable at 4 °C for only three weeks.

4. *Stock peroxidase.* Dissolve 100 mg of horseradish peroxidase (EC 1.11.1.7) (Sigma) in 100 mL of demineralized water. This solution is stable at 0 °C for six weeks.

5. *Working solution p-hydroxyphenylacetic acid/peroxidase.* Dilute 5 mL each of stock solutions 3 and 4 to 500 mL with demineralized water. This gives a working solution containing, per liter, 50 mg of *p*-hydroxyphenylacetic acid and 10 mg of peroxidase.

6. *Galactose oxidase.* Prepare this freshly each day by dissolving 400 U of galactose oxidase (Worthington Chemical Co., Freehold, NJ 07728) in 100 mL of demineralized water. The enzyme from this manufacturer is marketed as a powder in individual vials, each containing 400–450 U of activity. Store the powder at −80 °C. The K_m of galactose oxidase is 0.20 mol/L (*13*). Enzyme from other commercial sources showed low activity and gave unpredictable recoveries of galactose.

7. *Stock galactose standards, 1.0 g/L.* Dissolve 100 mg of D-galactose in 100 mL of saturated benzoic acid.

8. *Working galactose standards.* Dilute the stock solution with demineralized water to give standards of 20, 40, 80, and 120 mg/L. Prepare freshly each day.

9. *Sodium hydroxide.* Using carbonate-free NaOH and demineralized water, prepare 0.4 and 0.5 mol/L solutions.

10. *Zinc sulfate.* An aqueous solution of $ZnSO_4 \cdot 7H_2O$, 100 g/L.

11. *Wash solution.* To 410 mL of demineralized water, add 50 mL of 0.5 mol/L NaOH and 40 mL of reagent 10, shake, and filter. This wash solution between samples was necessary to provide a consistent background matrix of the same composition as the samples. We used the smaller volume of $ZnSO_4$ because 20% less $ZnSO_4$ precipitates from aqueous solution than from serum when equal volumes of $ZnSO_4$ and NaOH are used (*17*).

Procedure

Analyze the clear deproteinized filtrate by continuous-flow (see Figure 1), using two coupled enzymic reactions. The limiting reaction is the specific oxidation of galactose by galactose oxidase:

$$\text{D-Galactose} + O_2 \rightarrow \text{D-galactohexadialdose} + H_2O_2.$$

In the second reaction *p*-hydroxyphenylacetic acid acts as hydrogen donor for the peroxidase reaction; the product of this oxidation is fluorescent:

$$p\text{-Hydroxyphenylacetic acid} + H_2O_2 \rightarrow \text{fluorescent compound} + 2H_2O.$$

The manifold (Figure 1) is constructed from AutoAnalyzer modules (Technicon Instruments Corp., Tarrytown, NY 10591). The sample rate is 40/h, with a sample: wash ratio of 1:2.

Equal volumes of sample and the buffer (solution 2 above, pH 7.0) are mixed, then galactose oxidase and the working solution of *p*-hydroxyphenylacetic acid plus peroxidase are added. These react for 15 min (time-delay coil) at 40 °C. A longer delay will give only a small increase (<15%) in sensitivity.

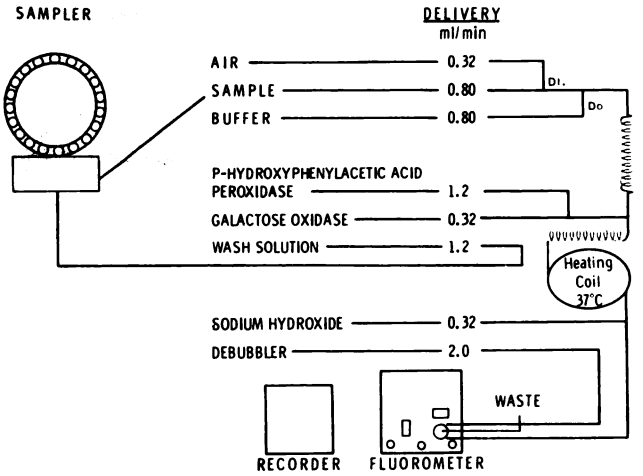

Fig. 1. The galactose manifold and flow diagram for automated analysis of galactose in concentrations 0–100 mg/L

Fluorescence is optimal at pH 11, which is achieved by adding 0.4 mol/L NaOH at room temperature. Fluorescence at pH 7.0 is approximately one-tenth of that at pH 11.0. The fluorescent compound is measured in a Fluorometer II (Technicon), fitted with a black glass flow cell (Technicon part no. 013-B008-01), with a Corning 7-60 primary filter (wavelength 365 nm) and combined secondary filter of Turner 2A and Kodak Wratten 47-B (wavelength 436 nm).

Analytical Variables

Recovery and precision were evaluated as follows. Plasma test samples containing expected concentrations of 10, 40, 70, and 100 mg/L were prepared by adding galactose to plasma from fasted subjects. Blood (100 mL) was drawn (with fluoride anticoagulant), and the plasma was separated, divided into five aliquots for multiple assay on different days, and stored at −20 °C until analysis. On each of five days, samples from each concentration were assayed in duplicate, four times in each run. Hence, 40 assays were performed on each galactose concentration. Between-duplicate precision, within-run precision, and day-to-day precision were assessed from these multiple analyses.

Results

For duplicate galactose standards of 10, 20, 30, 40, 50, 70, 90, and 120 mg/L, the regression equations for the percent transmission (y) vs galactose concentration (x) was $y = 2.77 + 11.54\,x$, with $r^2 = 0.998$, and standard error of 2.14. Excluding the 10 and 120 mg/L standards gives a virtually linear calibration for a defined working range of 20 to 90 mg/L. With this method, sample peaks reach 96–98% of a repeated, steady-state sampling of the same standard.

Recovery and Precision Studies (Table 1)

The upper portion shows the overall mean and its standard error for each concentration. Recovery percentages, based on expectations of 10, 40, 70, and 100 mg/L, averaged 96.5, 99.2, 101.0, and 98.5%, respectively.

The 40 assay results at each concentration form a twofold nested or hierarchical design, from which sample, run, and day variance components were estimated. In turn, these estimates were used to derive the standard deviations of replicate assays which represent sample-to-sample, within-run, and day-to-day precision. These standard deviations are summarized in

Table 1. Precision of the Assay for Galactose

	Expected concn, mg/L			
	10	40	70	100
Overall assay mean	9.7	39.7	70.7	98.5
SEM	0.66	0.60	0.90	0.51
SD of replicate assays:				
Sample-to-sample	0.74	1.51	0.87	1.30
Within-run	0.83	1.51	1.54	2.07
Day-to-day	1.66	1.98	2.43	2.17

the lower portion of Table 1. For example, for the 70 mg/L concentration, replicate assays between duplicate samples have a standard deviation of 0.87 mg/L, and replicate assays within the same run on one day have a standard deviation of 1.54 mg/L, and replicate assays on different days have a standard of 2.43 mg/L. As is common in such assays, standard deviations tend to increase with increasing concentration; however, the relationship is not proportional. Depending on concentration, CV's from sample-to-sample ranged from 1.2 to 7.7%, within-run from 2.1 to 8.6%, and day-to-day from 2.2 to 17.2%. The largest CV's were at the 10 mg/L concentration; the smallest were at the 70 or 100 mg/L concentration.

Illustrative Galactose Infusion Studies (Figure 2)

This shows the plasma galactose concentrations during infusion of 50 g/L galactose in a normal and a cirrhotic subject. The infusion rate was 60 mg of galactose per minute; a steady-state plasma concentration was reached at 80 min. In the cirrhotic patient, plasma galactose plateaued at 96 mg of galactose per liter; in the normal subject, the plateau concentration was 48 mg/L. Calculate the galactose clearance from the equation (*18*):

$$\text{Plasma clearance} = \text{infusion rate} / \text{steady-state plasma concentration}$$

Clearance was 1250 mL/min in the normal subject, and 630 mL/min in the cirrhotic patient. Reports on a series of such studies will be published elsewhere.

Discussion

Some Problems and Their Solution

In attempting the transition from measuring high concentrations of galactose in small samples, as described by Mason (*15*), to small concentrations in large samples, we identified two problems: interfering substances in blood totally inhibited the enzyme reaction in untreated samples, and the fluorometric method as previously described (*15*) was inadequately sensitive.

The principal substance in plasma interfering with the enzyme reactions was uric acid; analogous problems were encountered by Kreutzer (*19*) in the glucose oxidase/peroxidase determination of blood glucose. In whole blood, the most significant interfering substance was glutathione (*17*).

Initially, we attempted to remove these compounds with ion-exchange resins, an approach useful in measuring low concentrations of glucose in urine (*20, 21*). The interfering substances were removed by a strongly basic resin, with only a twofold sample dilution, but the method was tedious and only semiquantitative.

We could totally remove the interfering substances by alkali deproteinization with a heavy metal salt (*16*), but this procedure diluted the sample 10-fold. Improved assay sensitivity required to measure the 10-fold reduced galactose concentration was achieved by optimizing each reagent. In confirmation of Guilbault et al. (*22*), we found *p*-hydroxyphenylacetic acid improved the sensitivity by twofold as much as homovanillic acid, the other commonly used substrate, particularly at low concentrations. The excitation wavelength of the oxidized product of *p*-hydroxyphenylacetic acid is 317 nm and the emission wavelength, 414 nm. The optimum concentration of this substrate in the working solution was 50 mg/L; there was no further increase in sensitivity above or below this concentration.

The concentration of enzymes described were those we found optimal. The purity of the galactose oxidase also appeared to be critical to the assay, as previously discussed.

Sample pretreatment by zinc/alkali deproteinization resulted in salt precipitation within the analyzer tubing at two sites. First, calcium is not precipitated by $ZnSO_4$/NaOH, but does precipitate with a phosphate buffer at pH 7.0. Our use of a Tris buffer, which does not precipitate calcium, overcame this problem. Second, when an equal volume of $ZnSO_4$/NaOH wash solution was used, zinc precipitation occurred at the final alkalinization step. As mentioned earlier, preparation of the wash solution with 20% less $ZnSO_4$ has proved satisfactory without significantly altering the solution's pH.

Sample Collection

Blood samples collected without fluoride and left at room temperature for 2 h showed an average loss of 24 mg of galactose per liter. Satisfactory collection was achieved by drawing blood into fluoride/oxalate anticoagulant; placing immediately on ice; and separating plasma within 2 h. Under these conditions, less than 1.0 mg of galactose per liter is lost. The loss from the samples without fluoride was significant ($p < 0.005$) after 1 h and longer, compared with treated samples. The use of fluoride raises the objection that it may inhibit the analytic enzyme reactions as well as desired inhibition of the metabolism of galactose by erythrocytes. However, we have not found this to be the case. Plasma samples collected thus, with galactose then added, have given average analytical recoveries of 97%. Similarly, the glucose oxidase/peroxidase reaction is not inhibited when the sample is buffered at pH 7.0 (*17*).

Hemolysis and hyperbilirubinemia frequently interfere with both colorimetric and fluorometric analysis. These presented potential problems with our method, because fluoride/oxalate produces some hemolysis, and some of our patients with liver disease show hyperbilirubinemia. $ZnSO_4$/NaOH precipitates both hemoglobin and bilirubin from plasma, and we have had satisfactory recovery studies with serum bilirubin as high as 300 mg/L.

Advantages

Clearance rates of galactose from blood in the 0–100 mg/L range are dependent on liver blood flow, but previously no method had been available to measure such concentrations. The increased sensitivity of fluorometry for such an assay was demonstrated by Mason and his co-workers (*15*) in their galactosemia work. Their blood-spot filter-paper technique presumably minimized the problem of endogenous plasma substances interfering with the analytic enzyme reactions. Our requirement of measuring galactose concentrations in the 0–100 mg/L range compounded the problem. Fluorometry offers far superior sensitivity over colorimetric methods, and by optimizing the reagent concentrations, we have been able to utilize this sensitivity to meet our requirements. Pretreatment of samples by the Somogyi method removes not only the constant interfering substances for the peroxidase reaction, but also the variable interfering compounds such as bilirubin.

The metabolism of galactose by the erythrocytes merits further comment. Extrapolation of our in vitro data, in which we demonstrated 24 mg/L loss of galactose over 2 h, implies

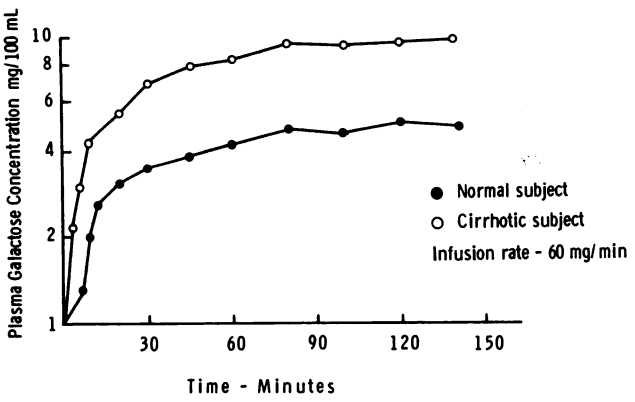

Fig. 2. Galactose infusion studies in normal and cirrhotic subjects

Both attain steady-state plasma concentration after 80 min. Calculation of clearance rate is given in the text

metabolism of 120 mg of galactose by the erythrocyte mass in 2 h (assuming a blood volume of 5 L), or 1 mg/min. In terms of our proposed clearance studies, infusing 50–100 mg of galactose per minute, this metabolism amounts to 1 to 2% of galactose clearance.

Galactose concentrations can now be measured in blood or plasma in the 0–100 mg/L range. This opens the way to more extensive evaluation of the clearance of galactose from blood at these concentrations, and hence the measurement of nutrient hepatic blood flow.

Addendum

Since we submitted this manuscript, one lot (No. 59E555) of galactose oxidase from Worthington has shown decreased sensitivity and stability. Subsequent production (lot no. 59P3437) has shown the same degree of sensitivity as described in the text. Evaluation of five further commercial sources of galactose oxidase has shown that the only other enzyme with the requisite sensitivity is available from P. L. Biochemicals, Milwaukee, WI 53205.

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