Automated Fluorometric Analysis of Galactose in Blood

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In galactosemia, prevention of mental retardation depends on early recognition of the disorder and institution of dietary restriction of galactose. We describe an automated fluorometric micromethod for galactose in whole blood spotted on filter paper. Galactose is oxidized by galactose oxidase to D-galacto-hexodialdose and H₂O₂ and measured as the highly fluorescent condensation product of homovanillic acid formed when H₂O₂ is acted upon by horseradish peroxidase. The procedure is 10-fold more sensitive than colorimetric procedures for galactose and is not hampered by the nonspecific fluorescence from endogenous NADPH that is encountered in methods in which galactose dehydrogenase is used. At a sampling rate of 40/h with a sample-to-wash ratio of 1/2, carryover is negligible, reproducibility is excellent, and 80% of steady state is achieved. Analytical recovery of added galactose was 95%. The method has the requisite sensitivity and accuracy for quantification of galactosemia and galactosuria in milkfed newborn infants and genetic evaluation of families of patients.

Additional Keyphrases: inherited disorders · diagnostic aids · continuous-flow analysis · screening · deficiency of certain hepatic enzymes · newborns

Transferase-deficiency galactosemia is an inborn error of metabolism with a reported prevalence of 1/5500 to 1/190 000 (1). Clinical and pathophysiological features of the disorder, which include failure to thrive and cataract formation, may lead to irreversible damage to liver and brain, and fatalities have been reported resulting from the intrinsic liver disease. If the condition is detected early in life and galactose and its major precursor, lactose, are removed from the diet, the untoward clinical consequences of the disorder may be prevented.

Classical galactosemia results from a deficiency of galactose-1-phosphate uridylyltransferase (EC 2.7.7.12), which catalyzes the second of the following sequence of reactions:

(1) Galactose + ATP → galactose-1-phosphate + ADP
(2) Galactose-1-phosphate + UDPglucose ↔ UDPgalactose + glucose-1-phosphate
(3) UDPgalactose ↔ UDPglucose

The respective enzymes involved are: (1) galactokinase (EC 2.7.1.6), (2) galactose-1-phosphate uridylyltransferase, and (3) uridine diphospho-galactose-4'-epimerase (EC 5.1.3.2). After a galactose-containing meal, galactose-1-phosphate accumulates intracellularly and galactose concentrations increase in blood and urine (2). The increase in galactose-1-phosphate intracellularly is thought to be toxic and related to the pathological changes observed in classical galactosemia (3, 4). Deficiencies of galactokinase or uridine diphospho-galactose-4'-epimerase lead to much less severe clinical symptoms generally than transferase-deficiency galactosemia (5, 6), but early detection of these disorders is likewise important from the standpoint of early institution of a galactose-restricted diet in an effort to prevent possible irreversible pathological changes.

The need for suitable methods for early detection of the various forms of galactosemia in large populations has prompted development in our laboratory of a sensitive automated fluorometric micromethod for galactose in whole blood spotted on filter paper. The method has the requisite specificity for galactose and is approximately 10-fold as sensitive as the colorimetric galactose oxidase assay reported by Tengstrom (7). Moreover, the procedure is not hampered by nonspecific fluorescence of endogenous NADPH, a rather serious problem in a method involving galactose dehydrogenase (8). In cases where an above-normal galactose concentration is demonstrable, the method could be used in conjunction with the automated fluorometric method of Frazier and Summer (9) for assay of galactose-1-phosphate uridylyltransferase activity, to distinguish transferase-deficiency galactosemia from other variant forms of this disorder.

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The similarity of the procedure we have developed for galactose to the automated method for glucose reported by Seiter et al. (10) from this laboratory offers the possibility that the two assays could be combined into a single manifold for simultaneous determination of both sugars from the same whole-blood sample spotted on filter paper. This is particularly important because severe hypoglycemia may be observed as a part of the clinical symptomatology in galactosemia, and, in addition, galactose could be determined at the same time as glucose in urine samples in cases where the dipstick test (Clinitest) is positive.

Materials and Methods

Principle

The present method for galactose is basically similar to the automated fluorometric procedure for glucose in whole blood devised earlier in our laboratory (10), an adaptation of the manual method of Phillips and Ele- vich (11). Galactose is determined by measuring the highly fluorescent complex produced from the condensation of homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid) following the action of horseradish peroxidase (EC 1.11.1.7) on the H₂O₂ generated when galactose is acted upon by galactose oxidase (EC 1.1.3.9):

\[
\text{D-Galactose + O}_2 \xrightarrow{\text{galactose oxidase}} \text{D-galacto-hexodialdose + H}_2\text{O}_2
\]

\[
\text{Homovanillic acid + H}_2\text{O}_2 \xrightarrow{\text{peroxidase}} \text{fluorescent product + H}_2\text{O}
\]

Reagents

Stock phosphate buffer: Prepare a phosphate buffer (0.1 mol/liter, pH 7.0) by dissolving 5.38 g of monobasic sodium phosphate and 16.35 g of dibasic sodium phosphate in 500 ml of distilled water. Dilute this solution to 1000 ml with distilled water. The buffer is stable for at least six months when stored at 4 °C.

Working phosphate buffer: Prepare a 10 mmol/liter phosphate buffer (pH 7.0) just before use by diluting the stock buffer 10-fold with distilled water and adding 0.5 ml of a 300 ml/liter solution of Brij 35 surfactant (Fisher Scientific Co.) per liter.

Stock homovanillic acid solution: Dissolve 15 mg of 4-hydroxy-3-methoxyphenyl acetic acid (Sigma Chemical Co., St. Louis, Mo. 63178) in a total volume of 50 ml of distilled water. This solution is stable at 4 °C for at least six weeks.

Peroxidase solution: Dissolve 50 mg of horseradish peroxidase (Type II, Sigma) in distilled water and dilute to a total volume of 50 ml. This solution is stable at 4 °C for at least six weeks.

Working homovanillic acid/peroxidase solution: Prepare a solution containing, per liter, 15 mg of homovanillic acid and 10 mg of peroxidase by diluting the stock homovanillic acid solution and the stock peroxidase solution 100-fold with distilled water.

Galactose oxidase stock solution: Prepare a solution of galactose oxidase (Worthington Chemical Co., Freehold, N. J. 07728) containing 100 units of activity per milliliter of distilled water. This solution is stable at 4 °C for at least six weeks.

Galactose oxidase working solution: Just before use, dilute 2 ml of the galactose oxidase stock solution to 100 ml with distilled water.

Benzoi acid solution: Prepare a saturated solution of benzoic acid by dissolving 2.9 g of benzoic acid in 1 liter of distilled water. This solution is stable indefinitely at room temperature.

Galactose standards: Prepare a stock solution of 250 mg of D-galactose per 100 ml of saturated aqueous benzoic acid. Dilute aliquots of this stock solution with saturated benzoic acid to the desired concentrations (5 to 25 mg/liter). This stock solution is stable for at least six months at 4 °C.

Galactose Manifold

The manifold consists of "AutoAnalyzer" modules as shown in Figure 1 (Technicon Instruments Corp., Tarrytown, N. Y. 10591). Analyses were done at a rate of 40/h, with a sample-to-wash ratio of 1/2. Samples were dialyzed against the working buffer and mixed at 37 °C with the enzyme and homovanillic acid/peroxidase solutions. The mixture was allowed to react for 15 min in a 40 °C time delay coil and then made alkaline with 0.4 mol/liter sodium hydroxide. The fluorescence of the oxidized homovanillic acid was measured in a fluoronephelometer (Technicon), with use of Corning 7-60 primary and Kodak Wratten 47-B and 2-A secondary filters, with an 85-W mercury lamp (Technicon No. 633-0037-01).

Experimental Procedures

Preparation of samples: Capillary blood was sampled by pricking the finger or heel. Blood was allowed to fall freely onto each of four circles measuring 0.95 cm in diameter marked on S&S No. 903 filter paper (Schleicher and Schuell Co., Keene, N. H., 03431). Each circle was saturated completely with blood. It is important to verify saturation of the circles with blood after obtaining the sample by inspecting both sides of the filter paper. The samples of blood on filter paper were then dried at room temperature for at least an hour and not more than 2 h, placed in 5 × 8 cm sealable polyethylene plastic bags (KCL Corp., Shelbyville, Ind. 46176) and stored at −20 °C until submitted to the laboratory for analysis. In some studies blood was taken in heparinized evacuated blood-collection tubes (Vacutainer tubes; Becton-Dickinson, Rutherford, N. J. 07070) by venipuncture and spotted on the filter paper forms with a disposable Pasteur pipette. These samples were sealed in plastic bags and stored at −20 °C or at room temperature, depending on the experimental conditions used.
Before analysis, each sample of dried whole blood was punched out of the filter paper with a circular hole-puncher measuring 0.95 cm in diameter (about 25 μl of whole blood) and eluted in a capped 2-ml conical AutoAnalyzer cup (Technicon) for 30 min with 1.0 ml of saturated aqueous benzoic acid containing 10 mg of galactose per liter.

**Tests for specificity:** To test the specificity of the method, we prepared 5–25 mg/liter standards of glucose, lactose, fructose, and galactose-1-phosphate. These concentrations are equivalent to 200–1000 mg/liter in blood. Automated analysis at 40/h (1/2) was performed following analysis of galactose standards of equal concentration.

**Analytical recovery of galactose:** A 10-ml sample of whole blood was collected in heparin and divided into two equal parts. To one part, galactose was added to make a solution with a concentration of 1.40 g/liter; no galactose was added to the other part. The two parts were mixed in different proportions to obtain samples with added galactose concentrations of 0.20, 0.40, 0.60, 0.80, 1.00, and 1.20 g/liter. The unmixed parts containing 0 and 1.40 g of galactose per liter were also used in this experiment. Twelve blood spots containing about 25 μl of whole blood were prepared from each of the samples containing different galactose concentrations. After drying, these were divided into three batches consisting of four filter-paper blood-spot samples of each concentration. Batch one was sealed in a plastic bag and immediately frozen at −20 °C until galactose was determined. Batch two was dry-autoclaved for 1 h at 110 °C, then sealed in a plastic bag and frozen at −20 °C. Batch three was sealed in a plastic bag and exposed to room temperature for one week before galactose was determined.

**Galactose tolerance test:** A galactose tolerance test was performed on each of three subjects: a normal man, S.C.; a woman heterozygous for classical galactosemia, J.A.; and her 12-year-old galactosemic son, S.A. Each subject fasted for 12 h before ingesting a galactose load of 15 g/m² body surface. Blood was collected just before the load, and 30, 60, 90, 120, and 240 min later by venipuncture (venous blood) or by finger prick (capillary blood). Part of each sample of capillary or venous blood (25 μl) was added directly to the benzoic acid eluent in an AutoAnalyzer cup for immediate determination of galactose and glucose; the rest was spotted on filter paper, dried, sealed in a plastic bag, and frozen at −20 °C until analyzed the next day. The blood glucose concentrations of the galactosemic subject were monitored during the study by the method of Seiter et al. (10).

**Results and Discussion**

For screening purposes filter-paper specimens containing about 25 μl of whole blood are punched out and are diluted 40-fold with 1 ml of saturated benzoic acid containing galactose in a concentration of 10 mg/liter. Because whole blood normally contains no measurable galactose, we added this galactose to the diluent in order that each sample would be represented by a peak on the recorder tracing. For most samples, peak heights are slightly less than the 400 mg/liter standard because about 5% of the galactose in the diluent is apparently metabolized by enzyme activity remaining in properly handled blood spots. This observation, however, presents no serious drawback to use of the method as a means of screening or for quantifying blood galactose.

Figure 2 shows a profile of galactose standards (5–25 mg/liter) equivalent to concentrations in whole blood of 200, 400, 600, 800, and 1000 mg/liter, which were run at a rate of 40/h with a sample-to-wash ratio of 1/2. Carryover is negligible, reproducibility is excellent, and 80% of steady state is achieved.

Glucose, lactose, and fructose in concentrations of 200 to 1000 mg/liter produced no detectable fluorescence or interference in this method, but galactose-1-phosphate produced about 10% as much fluorescence as galactose. This is consistent with the report of Avigad et al. (12) on the specificity of galactose oxidase from...
Polyporus circinatus (also called Dactylium dendroides), the source of enzyme used in this study. However, the effectiveness of this method as a screening procedure for galactosemia is not hindered by the interference from galactose-1-phosphate, because detectable concentrations of this metabolite are reached only in galactosemic subjects.

Galactose recoveries were determined for samples frozen immediately after preparation, samples autoclaved and then frozen, and samples exposed to room temperature for a week before analysis. The mean analytical recovery of galactose from samples frozen after preparation was 95.5% (Table 1), that for samples exposed to room temperature for one week was 79.3%, and that for galactose from autoclaved samples averaged less than 25%.

Results of oral galactose tolerance tests in three subjects are shown in Figure 3. The curves are characteristic of those reported for transferase-deficiency galactosemics, the heterozygous state, and normal subjects (13, 14). There was no discrepancy between the galactose values obtained with venous and capillary blood, or between the values obtained with samples spotted on filter paper and samples of whole blood (25 μl) added directly to the eluent in sample cups. These results demonstrate that this method can be used effectively as a diagnostic tool when galactose tolerance tests are administered in studies on patients suspected of having a deficiency of certain liver enzymes, e.g., transferase-deficiency galactosemia or Type I glycogen storage disease (Von Gierke’s disease).

The automated method for galactose that we describe here is rapid, specific, and sensitive enough to measure this carbohydrate in 25-μl samples of whole blood spotted on filter paper. By coupling this method with the glucose procedure described by Seiter et al. (10) one should be able to effect substantial cost benefits in assessing the clinical status of newborn infants and patients suspected of having galactosemia. The initial cost and operating expense of the combined galactose-glucose methods would be much less than two separate manifolds for each procedure, thus making such a combination advantageous for central laboratories that do screening and diagnostic confirmation of inborn errors of metabolism.

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References


**Table 1. Galactose Recovery from Blood Spots**

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<tr>
<th>Expected mg/liter</th>
<th>Found mg/liter</th>
<th>Recovery, %</th>
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<tr>
<td>0</td>
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<td>—</td>
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<tr>
<td>200</td>
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<tr>
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<td>1394</td>
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**Fig. 3. Galactose values for blood of a transferase deficiency galactosemic subject (S.A.), a subject heterozygous for transferase deficiency galactosemia (J.A.), and a normal subject (S.C.) after administration of an oral galactose load (15 g/m² of body surface)