Rapid Method for Screening for Galactosemia and Galactokinase Deficiency by Measuring Galactose in Whole Blood Spotted on Paper

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An inexpensive automated fluorometric technique for blood galactose determination has been modified to use dried blood spots available through phenylketonuria screening programs. The method is more efficient because of its high capacity (≈85,000 tests a year), low cost (≈$3,000 a year in reagents), and high specificity (enzymatic method).

Additional Keyphrases: AutoAnalyzer • mass screening • disorders of galactose metabolism • fluorometry

Two major metabolic disorders are associated with galactose metabolism: galactokinase (ATP:d-galactose-1-phosphotransferase, EC 2.7.1.6) deficiency and galactosemia (1) secondary to a deficiency in galactose-1-phosphate uridyl transferase (UTP:a-d-galactose-1-phosphate uridyltransferase, EC 2.7.7.10) activity. One case reported in 1972 (2) described a third enzymatic (UDPGlucose 4-epimerase, EC 5.1.3.2) deficiency related to galactose metabolism. The clinical manifestations in galactosemia may vary in severity from perinatal death to mental retardation, cataracts, and hepatic cirrhosis; cataracts may be the only symptom of galactokinase deficiency. The infant reported (2) with the epimerase deficiency was apparently healthy.

Abnormally high concentration of galactose in the blood of milk-fed newborns is a biochemical manifestation of these disorders. We propose a modified technique (3) adapted to dried blood spots, which is rapid and sensitive.

Another automated method for blood galactose determination has already been proposed (4). It requires blood spots of 14-mm diameter, much bigger than those usually collected for phenylketonuria screening (5, 6). The Guthrie test based on bacterial inhibition (7) also measures blood galactose and remains an alternative procedure to the one presented in this paper. Other techniques for the screening for galactosemia depend on measurements of uridyltransferase activity by use of dried blood on paper (8, 9). Unfortunately the enzyme is frequently inactivated (by alcohol, heating, etc.) during transport or at the time of collection, and the number of falsely positive results has been relatively high in our experience. The technique of Hochella and Hill (9) gave us a subject-recall rate of 3%, which is in agreement with their published results. In a centralized laboratory making phenylalanine and tyrosine determination on each of 100,000 newborns per year, the clerical work involved in recalling this percentage of subjects is prohibitive and costly.

The technique we propose is suitable for galactose determination from blood spots on the usual phenylketonuria-detection cards. It has the advantage of screening for galactokinase deficiency as well as galactosemia, and the number of false positives is very low (≈1:6,000).

Material and Methods

Apparatus

A standard “AutoAnalyzer” (Technicon Corp., Tarrytown, N. Y. 10591) is used, including a Technicon Fluorometer II. The sample are analyzed at a rate of 60 per hour, with a 1:2 sample-to-wash ratio.

A Technicon “type C” membrane is used in the dialyzer and is changed every day. The delay coil is 26 m long, 1.6 mm i.d.

Reagents

Saline. Dissolve 9 g of NaCl in water. Add 1 ml of “Brij 35” (30 g/100 ml) and dilute to 1 liter with water.

Tris buffer [tris(hydroxymethyl)aminomethane], 0.1 mol/liter, pH 8.6 at 25°C. Dissolve 12.1 g of Tris in water. Add 122 ml of 0.2 molar hydrochloric acid and dilute to 1 liter with water. Add 1 ml of “Brij 35.”

Coenzyme. β-Nicotinamide adenine dinucleotide (β-NAD·3H₂O; Sigma Chemical Co., St. Louis, Mo. 63118). Dissolve 200 mg in 100 ml of Tris buffer. Prepare freshly each day.

Enzyme. Galactose dehydrogenase (d-galactose: NAD oxidoreductase, EC 1.1.1.48). Dilute the
commercial suspension (Sigma) in 100 ml of a 20 g/100 ml solution of ammonium sulfate to obtain a final activity of about 10 U/100 ml. Prepare freshly each day, and keep this solution below 4°C.

Washing solution. Dissolve 12.1 g of Tris in 1 liter of water.

Eluent. Dissolve 12.1 g of Tris and 3.9 mg of d-(+)-galactose in 1 liter of water (a quantity equivalent to 10 mg of galactose per 100 ml is added because there is no detectable galactose in the blood of normal infants; a peak for each sample is thus necessary for numbering purposes).

Galactose standard curve. Prepare a stock solution equivalent to 100 mg/100 ml of galactose in the blood by dissolving 39.0 mg of galactose in 1 liter of Tris buffer. Appropriate dilutions, taking into account that there is a 10 mg/100 ml equivalence in the eluent, are made for standards ranging from 20 to 80 mg/100 ml of galactose.

Procedure

Principle. The principle of the method lies in the fluorometric determination of NADH produced by the following reaction:

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\text{Galactose} + \text{NAD} \rightarrow \text{Galactonolactone} + \text{NADH} + \text{H}^+ 
\]

Setting the instrument. With the AutoAnalyzer system arranged according to the diagram in Figure 1, run the reagents for about 20 min to establish the baseline and then run a series of standards (20, 40, 60, and 80 mg/100 ml), each standard being separated from the next by a cup of Tris buffer. Transmittance should be at least 80% for the 100 mg/100 ml galactose standard if sensitivity and resolution are to be good when the samples will be run.

Preparation of samples. Collect blood spots on Schleicher and Schuell No. 903 C filter paper. Punch a disc 7.94 mm (\%) in.) diameter from the spot and transfer it to elute the blood from it by gentle shaking in 0.5 ml of the elution solution during 30 min. Then discard the disc, and samples are ready to be analyzed for galactose with the AutoAnalyzer. Care must be taken to adjust the Sampler probe a few millimeters from the bottom of the sample cup to avoid clogging from the paper fibers that settle out.

Results

Nonspecific fluorescence. Although there is no detectable galactose in the blood of normal infants (4), each sample gives a small peak, reflecting a small nonspecific fluorescence inherent to the technique. Depending on the actual activity of the commercial enzyme preparation, the relative nonspecific fluorescence may vary from one day to another. The nonspecific peaks illustrated in Figure 2 gave a mean of 8 mg/100 ml and a standard deviation of 1.8 for apparent galactose in 36 samples the day it was recorded.

This nonspecific fluorescence, which is almost constant from one sample to another, permits samples to be identified by numbering the peaks. Nevertheless, to each sample and standard is added galactose (final concentration, 10 mg/100 ml), which allows the rate of analysis to be increased to 60 per hour without serious loss of resolution.

Without added galactose, sample peaks that are low and do not return to baseline at the 60 per hour rate are sometimes difficult to identify. The heavy line at 15% transmittance in Figure 2 should then be considered as the real baseline because of this added galactose.

Sometimes the nonspecific fluorescence may be quite high in a given sample, but on repeated determination returns to normal. A positive result is considered confirmed only after at least two determinations.
Galactose stability. Peaks a and b in Figure 2 are determinations from blood spots, made after adding 40 and 80 mg, respectively, of galactose per 100 ml of blood. They were analyzed five days after they were made, which is the mean transit time (time between the blood collection and the analysis) for our screening program. The results for samples a and b were 31 and 57 mg/100 ml, respectively. About 30% of the galactose is evidently lost during this time at room temperature, in agreement with Tengström's results (4). This galactose instability prompted us to set a low threshold, 20 mg/100 ml, above which specimens would be recalled.

Cases detected. After 10 months of operation, 60,000 tests have been done. Ten specimens had a galactose concentration greater than 20 mg/100 ml, eight being between 20 and 40 mg/100 ml on their first test but negative on subsequent recalling. Values for other two samples were above 80 mg/100 ml on their first test. One is still under investigation; the other, who has been diagnosed as having galactokinase deficiency, was put on a restricted galactose diet at 20 days of age and has shown no symptoms since.

Discussion

With this rapid method for detection of galactosemia and galactokinase deficiency, suitable for mass screening, 85,000 tests a year can be done on one AutoAnalyzer. The method presents very few false positives (about 1 in 6,000).

The method was designed for mass-screening purposes, so the goal was to detect cases presenting high blood galactose concentration at a relatively low cost rather than to meet the usual fine requirements of accuracy needed in research protocols. Reagents cost about 3¢ per test, and only one technician is needed to prepare the samples and to operate the instrument. After 60,000 consecutive tests, not one case has been reported as having been missed by our program.

For more security, the threshold can be reduced to less than 20 mg/100 ml by adding more commercial enzyme thus increasing sensitivity and cost of the method. The limit of 20 mg/100 ml may be too high unless the specimen be obtained one or two hours after a feeding, since fasting levels of 3 mg/100 ml have been described in kinase and epimerase deficiencies. In our program until now, no effort has been made to obtain postprandial specimens.

The original method (4) not only required samples of inconvenient volume, but was four times as costly and 25-fold less sensitive.

It must be remembered that because of nonspecific fluorescence, it should not be decided that the test is positive without at least a second determination.

We believe this low-cost, high-capacity method will be useful in determining the incidence of galactose disorders in different populations.

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References

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