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Colorimetric Determination of Galactose and Galactose-1-Phosphate from Dried Blood

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A colorimetric microassay for the simultaneous quantitative determination of galactose (Gal) and galactose-1-phosphate (Gal-1-P) in dried blood spots is described. An enzymatic reaction involving alkaline phosphatase (EC 3.1.3.1) and galactose dehydrogenase (EC 1.1.1.48) produces NADH, which is coupled with diaphorase (EC 1.8.1.4) and iodonitrotetrazolium violet (INT). The colourless INT is converted to a formazan of red colour the intensity of which is quantitated either photometrically by a microplate reader or determined visually with sufficient sensitivity for screening purposes. We evaluated the assay on 200,000 blood samples in a newborn screening program, and were able to distinguish between classical and milder forms of galactosemia with ease.

KEY WORDS: galactosemia; colorimetry; newborn screening.

Introduction

Newborn screening for defects in galactose metabolism is important for early diagnosis and effective treatment of this group of hereditary metabolic disorders.

The measurement of galactose (Gal) and galactose-1-phosphate (Gal-1-P) concentration in dried blood spots is used to screen for defects of three enzymes of galactose metabolism: D-galactose-1-phosphotransferase (EC 2.7.1.6), alpha-D-galactose-1-phosphate-uridylyl-transferase (EC 2.7.7.12) and UDP-glucose-4-epimerase (EC 5.1.3.2). The screening test has to fulfill several requirements: it must be sensitive, quick, inexpensive and of high clinical resolution. Most neonatal screening laboratories use time-consuming and semiquantitative microbiological tests. Several authors (1–3) recommend a very sensitive, rapid and quantitative assay using the NAD^+ dependent galactose dehydrogenase (GADH) reaction, but expensive apparatus is needed.

We developed a colorimetric method using simple equipment for the quantitative determination of Gal and Gal-1-P. To release Gal from Gal-1-P, alkaline phosphatase (AP) was incubated with GADH. In a second step, NADH reacted with diaphorase and iodonitrotetrazolium violet (INT) producing formazan:

$$\text{galactose-1-phosphate} + H_2O \xrightarrow{AP} \text{galactose} + \text{phosphate}$$

$$\text{galactose} + NAD^+ \xrightarrow{GADH} \text{galactono-lactone} + NADH + H^+$$

$$NADH + H^+ + \text{INT} \xrightarrow{\text{diaphorase}} NAD^+ + \text{formazan (red colour)}$$

This method was tested on 200,000 samples.

Materials and methods

Reagents

The following solutions were used: Tris/HCl, 0.2 M, pH 8.6 (No. T-1503); nicotinamide adenine dinucleotide (β′-NAD^+), 45 mM (No. N-1511); p-iodonitrotetrazolium violet, 2.2 mM (No. I-8377); alpha-D-galactose-1-phosphate dipotassium salt, Type II (No. G-0380); all these substances were obtained from Sigma Chemie GmbH, Deisenhofen, Germany. In addition, $MgCl_2$*$6H_2O$, 20 mM (Art. 5833) was obtained from Merck, Darmstadt, Germany.

AP (calf intestine), suspension 5 mg/mL, specific activity 400 U/mg (No. 108146); and β-GADH (*Pseudomonas fluorescens*), suspension 1 mg/mL, specific activity 5 U/mg (No. 104973), were both purchased from Boehringer Mannheim, Germany. Diaphorase (*Clostridium kluyveri*), lyophilized powder 17 U/mg solid, specific activity 31 U/mg (No. D-2381) was from Sigma Chemie; 53.4 U was dissolved in 3 mL buffer containing 0.2 M Tris/HCl pH 8.6, 0.29 M KCl and 250 mg/L BSA.

Reaction mixture I for 300 samples consisted of 17 mL Tris/HCl, 4.5 mL NAD^+, 2.1 mL $MgCl_2$, 18.9 μL AP and 450 μL GADH. Reaction mixture II for 300 samples consisted of 3 mL INT with 3 mL diaphorase in the buffer described above.

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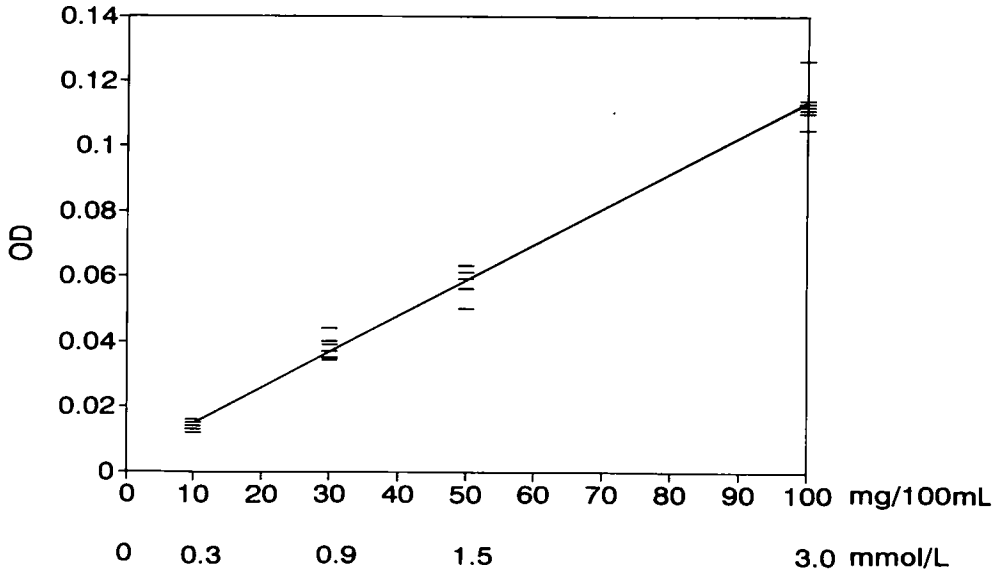

Figure 1—Regression line of galactose-1-phosphate standards with absorbance at 492 nm (OD).

DRIED BLOOD STANDARDS

Heparinized blood from healthy adults was diluted with a stock solution of Gal-1-P and Gal to yield final concentrations of 0.3, 0.9, 1.5, 3.0 mmol/L and 0.6, 1.7, 2.8, 5.6 mmol/L (10, 30, 50, 100 mg/dL) respectively. The blood standards were spotted on filter paper and dried at room temperature for 3 h. The standards remained stable for 4 weeks at 4 °C as already observed by Orfanos *et al.* (4).

DRIED BLOOD SAMPLES

Blood was collected from newborns on the fifth postnatal day and transferred to filter paper (Schleicher & Schüll, Dassel, Germany, No. 2992). The laboratory received the cards by mail and performed the test on the day of receipt. The blood samples were stored one month at 4 °C for possible repeat tests.

PROCEDURE

Discs of 4.3 mm diameter were punched into glass test tubes and placed for 23 min in a steam-bath for hemoglobin fixation. The discs were transferred into a microplate and 200 μL of 0.2 M Tris/HCl pH 8.6 was added to each well. The metabolites were eluted for 2 h on a shaker. A 170 μL aliquot of the eluate was transferred into another microplate. Eighty μL of reaction mixture I was added and the mixture was incubated for 1 h at 37 °C. Twenty μL of reaction mixture II was added and the mixture was incubated for a further 10 min in the dark at room temperature. The optical densities were measured using a microplate reader (Titertek Multiscan MCC 340, ICN Biomedicals GmbH, Meckenheim, Germany) at 492 nm with a reference wavelength of 620 nm.

CALCULATION

A linear regression curve was obtained by multiple analyses of Gal-1-P standards at concentrations of 0.3, 0.9, 1.5, 3.0 mmol/L, and of Gal standards of 0.6, 1.7, 2.8 and 5.6 mmol/L respectively (Figure 1). The linear regression and the coefficient of correlation were calculated daily using an on-line microcomputer. The patients data were obtained in terms of mg/dL on the basis of the regression curve.

Results

LABORATORY PERFORMANCE

The imprecision of the method was examined by assaying 10 samples of the same standard from several cards. For Gal-1-P the coefficient of variation in the run-to-run assay decreased from 15.75% at 0.3 mmol/L to 9.3% at 3.0 mmol/L, and in the within-run assay from 10.48% at 0.3 mmol/L to 4.81% at 3.0 mmol/L.

Gal-1-P standard at a concentration of 3.0 mmol/L was assayed from 1, 2, 3, and 4 discs all deriving from the same card. The absorption remained proportional to the number of discs and linear in the whole range. The correlation coefficient was greater than 0.982. The accuracy of the analysis has been confirmed by three other laboratories in a quality assurance program.

CLINICAL EVALUATION

More than 200,000 newborn infants have been tested. A transferase deficiency was detected in

three cases with Gal-1-P values greater than 3.0 mmol/L. There were no false negative or any technically false positive results.

Statistics were derived from 40,500 samples. The mean concentration of Gal-1-P was 0.15 mmol/L in such cases below the cut-off value of 0.74 mmol/L. The cut-off value was exceeded in 156 samples (0.39%) and the determination was repeated in duplicate. A second card was requested in 30 cases (0.07%) in whom the values for Gal-1-P were greater than 1.04 mmol/L. In these cases classical galactosemia was not found.

Discussion

Several authors have applied AP and GADH to measure total galactose in dried blood spots using a fluorometric assay (1,3,4). This paper describes a new colorimetric method exploiting a coupled reaction from galactose to formazan.

The advantages of the colorimetric over the fluorometric measurement are lower costs of the analytical equipment, fewer test repeats due to the lack of nonspecific fluorescence, and the possibility to inspect the assay visually with sufficient sensitivity for screening purposes; this gives screening centers which are not well equipped the chance to install this method.

Galactosemia is a rare disease and not all cases are treated successfully; it is essential that the assay is cheap enough to be acceptable for routine screening. Using the colorimetric assay, the detection of a positive case of galactosemia is not more expensive than the detection of a case of hypothyroidism of phenylketonuria.

The progress of galactosemia can be very severe; it is important that the primary test result is available on the day that the blood sample arrives. The overall assay time was 5 h and antibacterial substances did not interfere. Recently a faster hemoglobin fixation with organic solvents which reduced the assay time to 2.5 h has been described (3).

The method reported here is sensitive and precise enough to easily distinguish normal and affected newborn infants. With the test procedure of Yamaguchi *et al.* (3) even lower concentrations can be measured quantitatively, but this feature is of no value for screening of galactosemia.

The colorimetric assay for galactosemia has been tested for two years. It has fulfilled all requirements for a galactosemia screening test. It is sensitive, quick, inexpensive and of high clinical resolution. The establishment of this method in other screening laboratories can be recommended.

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