

A Screening Method for Biotinidase Deficiency in Newborns

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We describe a method for neonatal screening for biotinidase (EC 3.5.1.12) deficiency. Biotinidase activity is assessed colorimetrically from dried samples of whole blood spotted on the same filter papers as used in the neonatal screening for phenylketonuria. After the reaction, samples from normal infants are characteristically purple, whereas those from affected individuals are straw-colored. To confirm the deficiency, the enzyme is quantitatively assayed in additional blood spots or serum. A pilot study has been initiated with samples obtained by the Commonwealth of Virginia for phenylketonuria testing

Additional Keyphrases: *heritable disorders · colorimetry · screening*

Late-onset, biotin-responsive, multiple carboxylase deficiency (LMCD)³ is an inherited, autosomal recessive disorder characterized by skin rash, conjunctivitis, alopecia, ataxia, and often candidiasis and developmental delay (1, 2). Symptoms usually first appear during the first several postnatal months, but may not occur for several years. Patients with the disorder may exhibit ketolactic acidosis and organic acidemia, caused by deficient activities of the biotin-dependent enzymes propionyl CoA carboxylase (EC 4.6.1.3), pyruvate carboxylase (EC 4.6.1.1), and β -methylcrotonyl CoA carboxylase (EC 4.6.1.4) (2). These biochemical abnormalities can result in irreversible neurological damage or death. However, there is considerable clinical variability among patients from different families and among affected members of the same family (3).

We have recently shown (4) that the primary biochemical defect in LMCD is deficient activity of the enzyme biotinidase (biotin-amide amidohydrolase, EC 3.5.1.12), which catalyzes the removal of covalently bound biotin during carboxylase degradation (5, 6) and thus plays an important role in the recycling of this vitamin. The activity of the enzyme in the sera of patients with LMCD is almost undetectable (4).

In normal subjects biotinidase recycles biotin; whereas in biotinidase-deficient patients the biotin-salvage pathway is blocked, and high concentrations of dietary biotin are thus required to prevent or alleviate the symptoms of biotin deficiency (4). However, because the neurological and cutaneous manifestations of LMCD can occur in the absence of metabolic decompensation, there has usually been no reason to suspect the diagnosis at an early stage of the disease, nor could the diagnosis be confirmed without overt carboxylase deficiency (3). Consequently, the treatment of affected individuals was frequently not begun until irreparable damage had already occurred. Clearly, biotinidase deficiency satisfies several important criteria for neonatal screening: the

disease is not recognized clinically at birth; undiagnosed patients usually present with symptoms that may lead to mental retardation or death; and the administration of pharmacological doses of biotin provides a simple, inexpensive, and highly effective form of treatment (3). Although at least 22 cases of biotinidase deficiency have been confirmed enzymatically worldwide, the actual incidence of the disorder is unknown. The lack of consanguinity in previously reported families and the great variability in the clinical expression of the disease suggest that the gene frequency may well be similar to or greater than those of other genetic disorders of metabolism for which screening is currently performed (7). Moreover, as with phenylketonuria, the successful treatment of affected females may ultimately place their own offspring at risk of exposure to an unfavorable prenatal environment. At present, however, we believe this theoretical risk would be completely obviated by the continued treatment of such women with biotin throughout their lives and particularly during pregnancy. The purpose of our paper is to meet a final criterion for screening, i.e., the description of a simple, rapid diagnostic test for biotinidase deficiency; the same dried, blood-saturated paper discs currently used in most neonatal screening programs are utilized.

Materials and Methods

Sampling. Dried, blood-saturated, punched filter-paper discs (3 mm diameter) were obtained from screening cards (Schleicher and Schuell, Inc., Keene, NH 03431) used by the Commonwealth of Virginia for phenylketonuria testing.

Apparatus. The discs were automatically dropped by a Punch Indexer (Model VII; Fundamental Products Inc., North Hollywood, CA) into 250- μ L polystyrene sample cups (cat. no. 02-544-65; Fisher Scientific Co. Silver Spring, MD 20910) arranged in Plexiglass racks (260 \times 180 \times 6 mm) placed in "PKU trays" (cat. no. 029X; Buffalo Jewelry Case Division, Buffalo, NY 14210).

Reagents. *N*-Biotinyl-*p*-aminobenzoate (B-PAB) was prepared as previously described (4). *N*-1-Naphthylethylenediamine dihydrochloride, ammonium sulfamate, and *p*-aminobenzoic acid were obtained from J. T. Baker Chemical Co., Phillipsburg, NJ 08865; sodium nitrite from Eastman Kodak Co. Rochester, NY 14650; and trichloroacetic acid and potassium phosphate from Sigma Chemical Co., St. Louis, MO 63178. We tested the following drugs for interference with the colorimetric reaction: phenytoin (Parke-Davis, Morris Plains, NJ 07950), ampicillin (Bristol-Myers Co., Syracuse, NY 13201), gentamicin sulfate (Schering Corp., Kenilworth, NJ 07033), vitamin K₁ (Merck, Sharp & Dohme, West Point, PA 19456), penicillin G potassium (Pfizer Inc., New York, NY 10017), kanamycin sulfate (Beecham Laboratories, Bristol, TN 37620), sulfamethoxazole combined with trimethoprim, and sulfisoxazole (Hoffmann-LaRoche Inc., Nutley, NJ 07110).

Biotinidase activity determination. Biotinidase activity in discs was determined by the method of Knappe et al. (8), with the following modifications: 30 μ L of potassium phosphate buffer (50 mmol/L, pH 6.0) containing 150 μ mol of B-PAB and 5 mmol of EDTA per liter was added to each

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³ Nonstandard abbreviations: LMCD, late-onset, biotin-responsive multiple carboxylase deficiency; B-PAB, *N*-biotinyl-*p*-aminobenzoate.

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sample cup. The reaction trays were covered and incubated in a humidified chamber at 37 °C. After 16 h the reaction was stopped by adding 30 μ L of trichloroacetic acid (1.84 mol/L) to each cup. For color development we added sequentially, with a dispensing pipet, 30 μ L of each of the following reagents at 3-min intervals: sodium nitrite (14.5 mmol/L, prepared fresh daily), ammonium sulfamate (43.8 mmol/L), and *N*-1-naphthylethylenediamine dihydrochloride (3.86 mmol/L). Color development was complete 10 min after the addition of the last reagent. Those samples that became purple were considered to have biotinidase activity; those that remained straw-colored were considered to have little or none. Additional blood-sample discs from the latter infants were used for quantitative determination, as outlined by Wolf et al. (4), except that the concentrations of reagents were as described above, bovine serum albumin was excluded, and sample discs were placed in each of the assay tubes. Time-course and inhibitor studies were also conducted by this modification of the method of Wolf et al.

Results and Discussion

Incubation, development, and evaluation. From a 24-h time-course study (Figure 1) we determined that incubation should last at least 4 h. For routine determinations we used a 16-h period (overnight), but 8-h incubation also allows the detection of affected individuals. Trays should be read within 30 min of development, but the color is stable for at least 2 h.

Sample stability. Blood-saturated screening cards containing blood from normal and affected children were stored at room temperature and analyzed one, 30, and 60 days after collection. Test cards that had been stored for as long as 3.5 years were also obtained from the neonatal screening program of the Commonwealth of Virginia. Normal samples stored for no longer than 18 months still contained detectable biotinidase activity, whereas older samples and samples from affected infants did not. Routinely, samples can be tested anytime within seven days of collection. Consequently, and because the requirement of the screening procedure is for qualitative rather than quantitative assessment, slight losses of activity during storage will not alter the effectiveness of the method.

Interference. This colorimetric biotinidase assay depends

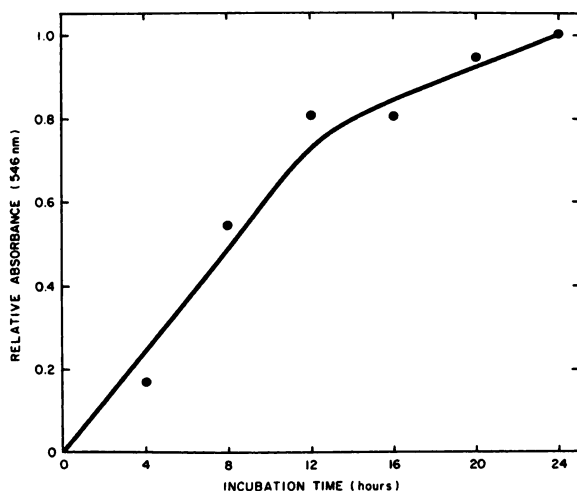


Fig. 1. The effect of incubation interval on color development in the biotinidase activity assay

Each point represents the mean of duplicate determinations made on five filter paper discs saturated with whole blood from a donor with normal biotinidase activity. The assay conditions were as described by Wolf et al. (4), modified as outlined in *Materials and Methods*. The absorbance reading for each incubation time is plotted as the fraction of the absorbance after 24 h

on the presence of a free primary aromatic amino group, or one that can be freed by hydrolysis (9). Therapeutic drugs administered to the mother immediately before parturition, or to the infant, might interfere by contributing to color formation; the former situation has not been encountered, however. In the developmental stages of this program, a sample of serum from a five-year-old child known to have biotinidase deficiency turned intensely purple, well above the upper limit of normal samples, suggesting the presence of biotinidase activity. The serum alone, without incubation with substrate, also turned purple during the development treatment. Subsequent inquiries revealed that the child was being treated for an ear infection with a sulfonamide that contained a primary aromatic amino group.

Two sulfonamides and other therapeutic drugs commonly used in the perinatal period were tested for their effect on color development. Predictably, the sulfonamides sulfisoxazole and a preparation containing trimethoprim and sulfa-methoxazole (pulverized and suspended in water) turned purple when developed in the absence of B-PAB (9). Phenytoin, ampicillin, gentamicin sulfate, vitamin K, penicillin G potassium, and kanamycin sulfate (each reconstituted according to manufacturers' recommendations and added to separate reagent tubes in aliquots of 100 μ L) gave no color when developed in the absence of substrate. None of these other compounds contributed to or detracted from color development when *p*-aminobenzoate was present. Because the concentrations of these medications greatly exceeded the expected concentrations in serum (10, 11), the possibility of interference is remote. Nevertheless, we recommend that infants whose samples turn either intensely purple or have little or no color be further investigated. If there is intense color, the serum sample should be developed in substrate-free buffer; subsequent color development would indicate the presence of interfering compounds. A quantitative assay of serum from infants yielding no activity in the screening test confirms the absence of activity; quantification of such samples will indicate the degree of enzyme deficiency.

Eight individuals referred to our laboratory have been diagnosed as having biotinidase deficiency (4). Although each had essentially undetectable biotinidase activity, we suspect that partial deficiencies may exist and could be missed by this method. We have also developed a radioisotopic assay for biotinidase (12), which has improved sensitivity and specificity. Compounds that interfere with the colorimetric assay do not affect this radioassay; this method can also be used to measure activity in samples from patients with putative biotinidase deficiency. The extra steps and expense involved, however, preclude the use of the radioisotopic assay for routine screening.

Cost-effectiveness. The incidence of biotinidase deficiency is unknown. However, in addition to the newly diagnosed individuals with biotinidase deficiency, several of the patients previously reported with LMCD have been shown to be biotinidase-deficient (K. Bartlett, J. M. Saudubray, and R. Baumgartner, personal communications). Most, if not all, patients with LMCD may be found to have biotinidase deficiency. Although some biotinidase-deficient patients exhibit organic aciduria and ketoacidosis, the disorder may occur without these symptoms. Because biotinidase deficiency is readily treatable, early identification of affected infants should enable the prompt initiation of biotin therapy. Excluding technical or administrative costs, we estimate that screening for this disorder would be extremely cost-effective, currently costing only \$0.01 to \$0.02 per test. A pilot study with samples obtained by the Commonwealth of Virginia for phenylketonuria testing has been initiated to determine the incidence of the disorder.

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Comparison of the Du Pont *aca* and Dow Methods for Determination of High-Density Lipoprotein Cholesterol

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We compared the Du Pont *aca* (phosphotungstate-enzymic cholesterol) and the Dow (dextran sulfate/Mg²⁺-enzymic cholesterol) methods for the determination of high-density lipoprotein cholesterol (HDL) and total cholesterol in serum from 113 patients. The *aca* results for both total cholesterol and HDL were significantly greater ($p < 0.0001$) than the Dow results, the *aca* method overestimating the HDL concentration (mean recovery 107.2% in serum samples with values assigned by the Centers for Disease Control). The precision of the *aca* method for HDL was essentially the same as that of the Dow method. Bilirubin (up to 0.17 g/L), hemoglobin (up to 4 g/L), and slight lipemia (triglycerides up to 5.4 g/L) did not interfere with the *aca* method.

Additional Keyphrases: variation, source of enzymic methods

Recently, a semi-automated enzymic method for high-density lipoprotein cholesterol (HDL)¹ became available on the Du Pont Automatic Clinical Analyzer (*aca*). The HDL is separated from other cholesterol by precipitation of apo B-lipoproteins with buffered phosphotungstate. We compared this method with a dextran sulfate/Mg²⁺-enzymic HDL method and evaluated the method for interference by bilirubin, hemoglobin, and lipemia. In addition, we compared the Du Pont and the Dow methods for the determination of total cholesterol (TC) in serum.

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¹ Nonstandard abbreviations: HDL, high-density lipoprotein cholesterol; TC, total cholesterol; CDC, Centers for Disease Control. Received January 13, 1982; accepted September 29, 1983.

Materials and Methods

We used an *aca* with computer II (Du Pont, *aca* Division, Wilmington, DE 19898) and a Model 250 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH 44074).

Reagents

Aqueous bilirubin, 0.5 g/L. Dissolve 12.5 mg of bilirubin (Standard Reference Material no. 916; National Bureau of Standards, Gaithersburg, MD) in 1 mL of dimethyl sulfoxide in a 25-mL volumetric flask. Add 2 mL of aqueous Na₂CO₃ (0.1 mol/L) and 18 mL of a 70 g/L solution of human serum albumin (Fraction V, cat. no. A2386; Sigma Chemical Co., St. Louis, MO 63178). Neutralize the solution (pH 7.0) by adding 2 mL of 0.1 mol/L HCl and dilute to volume with the human serum albumin solution. A bilirubin blank solution is prepared by the same procedure, but omitting the bilirubin.

Controls. To estimate precision, we used two control sera (Precilip, cat. no. 125067, Bio-Dynamics/bmc, Indianapolis, IN 46250; and Monitrol I, cat. no. B5103-1, Dade, Miami, FL 33152) and pooled human serum, prepared by the laboratory and stored at -20 °C.

Standards. We used aqueous cholesterol standards (Precision Cholesterol, no. 125512; Bio-Dynamics/bmc) for three-point calibration of the *aca* HDL and TC methods. One-point calibration of Dow HDL and TC methods was done with the Dow cholesterol standard (included in Dow reagent kit no. 46550).

Serum pools. Eighteen vials each of three serum pools were received frozen from the Centers for Disease Control (CDC), Atlanta, GA 30333. The HDL reference values were 0.265, 0.351, and 0.501 g/L, respectively. Each day of analysis we allowed three vials of each serum pool to thaw