A Qualitative Assessment of Biotinidase Deficiency*

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ABSTRACT

Screening programs for late-onset, biotin-responsive, multiple carboxylase deficiency (LMCD) detect colormetrically the presence of biotinidase activity in dried samples of whole-blood spotted on filter-papers as used in the neonatal screening of phenylketonuria. A sensitive and stable qualitative technique is described using 10 µl of serum that avoids problems associated with poor sample collection, improper drying of blood-spots and transient color development. The modified assay is timely and suitable for the clinical laboratory not involved in mass screening programs.

Introduction

Patients initially characterized as having “late-onset” biotin-responsive multiple carboxylase deficiency were shown by Wolf and colleagues in 198311 to have a deficiency of the enzyme biotinidase (biotinamide amidohydrolase, EC 3.5.2.12) in serum. This enzyme catalyzes the removal and release of free biotin which is recycled from biocytin (ε-N-biotinyl-L-lysine) or from biotinylated peptides produced by the normal degradation of biotin-dependent carboxylases12 (figure 1). Biotin is covalently bound through lysine to the carboxylases and functions as the CO₂ receptor in enzymatic carboxylation reactions. The four human carboxylases are crucial in fatty acid biosynthesis, gluconeogenesis, leucine metabolism and propionic acid oxidation.5

A deficiency of biotinidase activity is inherited as an autosomal-recessive trait.1 Deficient infants usually begin to show clinical signs several months after birth (onset at three to six months, but may be delayed up to 24 months).7 The untreated child may ultimately develop a variety of dermatologic, neurologic, metabolic, and immunologic abnormalities followed by coma or death (table I). The neurologic or cutaneous signs may occur in the absence of metabolic acidosis or
Biotinidase

**Biotinamide Amidohydrolase (Biotinidase) (EC 3.5.1.12)**

\[ \text{\varepsilon-N-biotinyl-L-lysine (peptides)} \rightarrow \text{biotin + lysine (peptides)} \]

\( \text{pH 6.0} \)

Figure 1. Biotinidase specifically cleaves the amide bond between the epsilon amino group of lysine and the carboxyl group of biotin.

detectable organic aciduria.\(^ {14} \) Therefore, definitive diagnosis requires the demonstration of the enzyme deficiency.

The purpose of our paper is to present a simple, rapid diagnostic test for biotinidase deficiency which utilizes 10 \( \mu l \) of serum in place of dried, blood-saturated paper disks currently used in pediatric and neonatal screening programs.

**Materials and Methods**

The biotinidase assay involves incubation of 10 \( \mu l \) of patient sera, standards, and positive controls in 30 \( \mu l \) of potassium phosphate buffer [(50 mmol per l, pH 6.0) containing 54 mg per l (140 \( \mu \)mol per l) of synthetic substrate \( N \)-biotinyl-\( p \)-aminobenzoate (or biotin-4-aminobenzoic acid*), 250 mg per l (3.6 \( \mu \)mol per l) bovine serum albumin, and 1.85 g per l (5.0 mmol per l) disodium ethylenediamine tetraacetic acid (EDTA)] at 37°C for four to 24 hr (table II). The incubations are carried out in a water bath using floating polystyrene reaction trays† containing 20 dimple wells each with 750 \( \mu l \) capacity and sealed with an adhesive covering to prevent evaporation. Sera from at least four patients can be run in duplicate with controls and reagent blanks.

Our method for serum biotinidase is a modification of a procedure described by Heard et al\(^ {4} \) that used a three mm blood-spot punched from a phenylketonuria (PKU) screening card (containing approximately three \( \mu l \) of blood). In the blood-spot procedure, the reaction trays are covered and incubated in a humidified chamber for 16 hr. In our experience, the final color was transiently detectable for approximately 30 min.

In the present procedure after incubation of sera and substrate, the reaction was stopped by adding to each reaction well 30 \( \mu l \) of 30 percent (1.84 mol per l) trichloroacetic acid. For color development, the following reagents were added sequentially with a dispensing pipet at three minute intervals: 30 \( \mu l \) of 1.0 g per l (14.5 mmol/l) sodium nitrate, 30 \( \mu l \) of 5.0 g per l (43.8 mmol per l) ammonium sulfamate, and 30 \( \mu l \) of 1.0 g per l (3.86 mmol per l) N-1-naphthylethylenediamine dihydrochloride (table III). Color development was complete within five minutes following addition of the last reagent. Those samples that became pink-purple were considered to have biotinidase activity; those that remained

<table>
<thead>
<tr>
<th>Clinical Findings in Biotinidase Deficiency</th>
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<tbody>
<tr>
<td><strong>Onset:</strong> Three to six months (may be delayed up to 24 months or longer)</td>
</tr>
<tr>
<td><strong>Dermatological:</strong> Skin rash, Alopoeica, Keratoconjunctivitis</td>
</tr>
<tr>
<td><strong>Neurological:</strong> Seizures and myoclonis, Hypotonia and tremor, Developmental delay, Hearing loss, Optic atrophy</td>
</tr>
<tr>
<td><strong>Metabolic and Immunologic:</strong> Acidosis and organic aciduria, Immune defects (candidiasis, recurrent infections)</td>
</tr>
</tbody>
</table>

* Sigma Chemical Co., St. Louis, MO 63178  
† Abbott Diagnostics, N. Chicago, IL, 60064
with a second repeat of the sample, a new serum sample is obtained and tested. If an abnormal result is obtained again, a fresh sample is procured for quantitative determination of biotinidase activity. Several quantitative procedures are available using fluorescence detection, high performance liquid chromatography with fluorescent detection, or radioassay using $^{15}$N-biotinyl-para-aminobenzoate or $^{14}$C-biocytin as the labeled substrate. Both kinds of assays have been found useful for quantitative measurement of biotinidase activity in normal peripheral blood leukocytes and fibroblasts.

**Evaluation of the Screening Method**

A reliable screening test should detect all affected individuals without false-positives. In general, false-positive tests have a smaller impact than do false-negative results. The economic and emotional costs of follow-up testing are small compared with failure to detect an infant or child with this disorder. The overall rate of occurrence of false-positive results (those requiring a second confirming assay) as reported by Heard et al was 0.09 percent. This rate compares favorably with false-positive rates (0.005 percent) of alternative procedures.

**Results and Discussion**

Biotinidase catalyzes the cleavage of biotinyl-p-aminobenzoate to biotin and para-aminobenzoate (PABA). After stopping the reaction with trichloroacetic acid, the amino group of PABA released via the action of biotinidase is diazotized and coupled with N-1-naphthylethylenediamine hydrochloride. The azo dye formed (pink-purple) is clearly visible to the eye. The final reaction color is stable and can be visually graded (table IV) or followed spectrophotometrically at 546 nm to give semi-quantitative results.

After incubation and development, samples are classified either as normal (having biotinidase activity) or abnormal, based on the color of the reaction mixture. Abnormal samples are subclassified into three groups, depending on whether the reaction mixture is pale-purple, very pale-purple, or straw-colored. If an abnormal result is obtained clear or straw-colored were considered to have little or none. The final color was stable for at least 48 hr at 4°C.

### TABLE III
Post-Incubation Development Procedure

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Time Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 μl 1.8 mol/l Trichloroacetate</td>
<td>0 min</td>
</tr>
<tr>
<td>30 μl 14.5 mmol/l Sodium nitrite</td>
<td>3 min</td>
</tr>
<tr>
<td>30 μl 43.8 mmol/l Ammonium sulfamate</td>
<td>3 min</td>
</tr>
<tr>
<td>30 μl 3.9 mmol/l N-1-Naphthylethylenediamine-dihydrochloride</td>
<td>3 min*</td>
</tr>
</tbody>
</table>

TABLE IV
Sensitivity and Qualitative Grading of Biotinidase Activity Using Para-aminobenzoic Acid

<table>
<thead>
<tr>
<th>Para-aminobenzoic Acid* (nmoles)</th>
<th>Intensity Grade†</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.00</td>
<td>++++++</td>
</tr>
<tr>
<td>3.00</td>
<td>++++</td>
</tr>
<tr>
<td>2.00</td>
<td>+++</td>
</tr>
<tr>
<td>1.00</td>
<td>++</td>
</tr>
<tr>
<td>0.50</td>
<td>+</td>
</tr>
<tr>
<td>0.20</td>
<td>+/-</td>
</tr>
<tr>
<td>0.12</td>
<td>-</td>
</tr>
<tr>
<td>0.10</td>
<td></td>
</tr>
</tbody>
</table>

*Para-aminobenzoic acid added per tray well.

percent to 0.52 percent) for other inborn errors of metabolism. The biotinidase screening test appears quite discriminating. In our laboratory, after 70 assays, false-negative results were not apparent. These data compare favorably with laboratories using the blood-disk method.4

Concern regarding transplacental passage of maternal biotinidase as a cause for false-negative results in the early neonatal period has not proven justifiable. For example, no blood biotinidase activity was detectable in a biotinidase-deficient infant with a heterozygous mother.4 Thus, it is unlikely that false-negative results will be a consequence of maternal biotinidase transfer to the newborn. However, transplacental transfer is an important consideration for other inborn errors, such as phenylketonuria where a metabolite can remain elevated for several days after birth.

False-negative results are more likely to occur as a result of drug interferences. For example, sulfonamide antibiotics react with the developing reagent to produce a pink-purple color.2 Therefore, an affected child receiving sulfonamides might appear as having enzyme activity. Fortunately, the presence of interfering compounds can be readily detected by running a blank serum in buffer without added substrate. This latter procedure is a routine component of our procedure.

Since there were no false-negative results, it can be concluded tentatively that the sensitivity of the test must approach 100 percent. The specificity is defined as the probability that the test will be negative when the disease is not present. By these criteria, the specificity after screening and repeat screening is greater than 99.99 percent.

Problems associated with poor blood spot collection technique, including incomplete saturation of a section of punched filter paper disk, are avoided using serum. Likewise, Heard et al have also reported some inconsistent results from disks dried improperly.5

Cost of Testing

The cost per test per child is relatively inexpensive since the laboratory technologist requires approximately one hour to complete four assays in duplicate. Using mass screening methods and punched disks, Heard et al5 have calculated costs at $0.24 per child assayed including analysis, reagent preparation, and communicating with the physician.

Summary

Biotinidase deficiency is readily demonstrable by colorimetric assay of the enzyme in serum using a synthetic substrate, N-biotinyl-p-aminobenzoic acid. This direct, functional assay has been modified and adapted in a low-cost screening method for neonates and children. The procedure can be completed in from four to 24 hr. Biotinidase deficiency testing fits the accepted criteria for inclusion in routine metabolic screening programs since it has significant incidence (1:40,000), excellent sensitivity (100 percent), and specificity (>99.99 percent). The deficiency usually remains unrecognized clinically until serious symptoms occur; it is life-threatening, and early treatment with biotin is effective.14
References


