Phenylketonuria: Contemporary Screening and Diagnosis

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ABSTRACT

Screening newborns for phenylketonuria (PKU) is a mandatory practice based on measuring a raised blood phenylalanine level. Many factors influence the rate of blood phenylalanine rise so that there are many pitfalls in detecting the 1:10,000 affected infant. About one percent of all babies tested proves to be "false positives." Two-thirds of those with persistent hyperphenylalaninemia prove to have classic PKU. Non-classic PKU with less intense, persistent hyperphenylalaninemia is due to different alterations in the enzyme, phenylalanine hydroxylase. Additionally, about one percent of the confirmed positive patients is due to either a defect in the synthesis or regeneration of the cofactor, tetrahydrobiopterin; these latter forms are not amenable to treatment with the low phenylalanine diet. Screening programs have developed directives regarding the timing and conditions for obtaining the specimens for testing. Specific confirmatory tests of those with positive results must be performed. Even so, about one in 70 affected babies is "missed," resulting in mental retardation, seizures, and neurologic deficits.

Introduction

Newborn screening for phenylketonuria using blood spots collected on filter paper was introduced in Massachusetts in 1962 and, with enabling legislation, was in use throughout the country within a decade. Last year, approximately ten million infants worldwide were screened for phenylketonuria. In Kentucky, the incidence of PKU is 1:10,000 of the approximately 60,000 screened each year.

In 1962 our understanding of the conversion of phenylalanine to tyrosine, the site of the "block" in PKU, was limited. Then, as babies began to be screened for PKU, many of those showing hyperphenylalaninemia did not prove to have the "classic" clinical form or biochemical pattern expected. This led to studies that have demonstrated that the oxidation of phenylalanine to tyrosine involves tetrahydrobiopterin as a cofactor (figure 1). More recent studies have shown that there are at least six molecular forms of the apoenzyme, phenylalanine hydroxylase, all with different levels of activity and clinical effect. In addition, three defects with a unique clinical pattern have been shown in the generation and recycling of the cofactor, tetrahy-
The conversion of phenylalanine to tyrosine is catalyzed by phenylalanine hydroxylase which uses the cofactor, tetrahydrobiopterin (BH$_4$). The cofactor is regenerated from the oxidized product quinonoid dihydrobiopterin (qBH$_2$) by dihydropteridine reductase.

Thus, physicians are dealing with multiple causes of hyperphenylalaninemia and with clinical effects that vary greatly in severity and in response to the traditional therapeutic low phenylalanine diet.

Measurement of only blood phenylalanine and its urinary metabolites is inadequate to assess the babies detected in our PKU screening programs; rather, additional metabolites must be measured in order to interpret a raised blood phenylalanine level. Screening for and diagnosis of PKU has become a process that involves measuring amino acids, cofactors, and cofactor products—a process that is frequently updated in light of new experience.

**Screening**

**METHODS**

In 1961, Robert Guthrie, a microbiologist and pediatrician, developed a semi-quantitative bacteriologic inhibition assay for phenylalanine.$^3$ The inhibitor, beta-2-thienylalanine, severely limits the growth of *Bacillus subtilis* in the presence of phenylalanine. The test proved to be simple and readily adaptable to filter-paper specimens of dried blood obtained from infants' heels. This test, with refinements, is the test still most widely used. In some states, however, an automated fluorometric method for measuring phenylalanine is utilized.$^5,6$ Compared to the Guthrie assay, it is more precise and quantitative and can measure phenylalanine down to zero.

**Positive Results**

Normal blood phenylalanine concentration is less than 0.12 mM (2.0 mg per dL). Since the mother clears increased blood phenylalanine in her affected fetus transplacentally, affected infants arrive with a normal blood phenylalanine. Each affected infant must have a sufficient dietary load, amount, and duration, of phenylalanine in order to increase signif-
icantly the blood phenylalanine level (>0.24 mM, >4 mg per dL). Normal breast milk or formula feedings for 48 hours usually is sufficient to raise the baby's blood phenylalanine sufficiently to trigger a positive result report.

Pitfalls

Even though screening for PKU using the metabolite blood phenylalanine is generally reliable, significant numbers of "false positive" cases occur; each requires evaluation. The "false positive" rate on babies with an initial raised blood phenylalanine level that later spontaneously declines to normal is approximately one percent of babies tested. Most of the "false positive" results occur in infants who are immature or prematurely born. In contrast, about one in 70 true cases is "missed" as a "false negative." In Kentucky, two missed cases are known to the author. One occurred as a labeling error in the newborn nursery, and one was never tested. Our experiences are similar to those of other states.7

Until a decade ago, it was standard practice for mothers and their babies to remain in the hospital for two to four days after delivery. Thus, almost all affected babies had a sufficient protein challenge to raise their blood phenylalanine to an abnormally high level. Changes in social and economic factors have promoted early discharge after birthing, raising the risk of "false negatives" because babies have not yet ingested a sufficient amount of protein to raise their blood phenylalanine level. However, some of this perceived risk is lessened because some elevation in blood phenylalanine occurs secondary to liberation of amino acids by the proteolysis that accompanies normal protein metabolism. Also, breast-fed infants receive colostrum with its elevated protein content in their first feedings. Even so, some babies born at term with PKU continue to go undetected on the initial screening test because they are tested too early or they are taking breast milk with its relatively low protein content.

Infants who are prematurely born are usually not fed within the first hours of life or even first weeks of life if ill. Ill infants born by Cesarean section frequently are not included in routine newborn care, so there is an administrative risk that they will not be tested. Thus, alternative testing time-tables have been developed to insure that all babies are tested at a time when there has been an adequate protein dietary load. In addition to lack of dietary protein challenge, ill infants frequently are administered antibiotics which may be in the infant's blood in sufficient concentration to impair bacterial growth in the Guthrie assay.2 In those states that utilize the automated fluorescent method, there is a risk of creating a "false positive" result. Some antibiotics spontaneously fluoresce. This problem has been encountered by us with ampicillin in the confirmation process.10

Remedial Steps

To address the screening pitfalls, many state PKU screening programs have attempted to compensate for these shortcomings by lowering the threshold for triggering a positive report from 0.24 mM (4 mg per dL) to 0.12 mM (2 mg per dL) and by calling for repeat or mandatory delayed testing on babies likely not to have experienced an adequate dietary protein challenge: breast-fed babies, babies discharged early from the hospital, and babies retained in our nurseries and intensive care units. The current administrative directives followed by us for newborn screening are listed.

- The hospital or other institution caring for infants 28 days or less of age and the attending physician or midwife shall cause to have administered to every infant in its care a blood test to detect PKU.
• In the event a baby is not born in a hospital or institution, the attending physician or midwife shall be solely responsible for causing such tests to be administered at no less than 48 hours or more than seven days of life.
• A capillary blood specimen shall be obtained from each infant before he or she leaves the hospital, regardless of the age of the infant. All infants screened prior to 48 hours of life shall be rescreened for PKU prior to three weeks of life.
• When an infant is transferred from one hospital to another during the newborn stay, the following rules apply. If the infant is 48 hours of age or more at the time of transfer to another hospital, testing for PKU shall be the responsibility of the sending hospital. It shall be the responsibility of the receiving hospital to ensure testing for PKU if the infant is less than 48 hours of age at the time of the transfer.
• A capillary blood specimen shall be obtained on the seventh (7th) day of life from an ill or premature infant or an infant receiving parenteral feeding still hospitalized on that day unless the particular infant has already been tested.
• A repeat capillary blood specimen shall be obtained from all infants who received transfusions prior to the initial screening. This specimen should be obtained 48 to 72 hours after transfusion.
• The capillary blood specimens required shall be obtained by a heel stick and the blood from the heel stick shall be applied directly to filter paper.

Repeat and Confirmatory Testing

Approximately one percent of the babies screened for PKU each year has a blood phenylalanine of greater than 0.12 mM (2 mg per dL) triggering a "positive" report (1:100). Of this relatively large number of patients with positive results, only an additional one percent will prove to have some form of PKU (1:10,000). An immediate sorting of the babies with positive results begins. For those that have only a modest increase in blood phenylalanine (0.12 to 0.36 mM or 2 to 6 mg per dL), a repeat specimen is sent to our laboratory where blood phenylalanine is measured by the alternative fluorometric technique.9,13 For those babies with a blood phenylalanine greater than 0.36 mM (6 mg per dL), immediate referral to our clinic is requested. For those babies with a persistent mild elevation (0.12 mM to 0.36 mM or 2 mg per dL to 6 mg per dL), repeat testing may be requested or the baby may be evaluated in our clinic. A liaison is maintained with the State Laboratory so that little time is lost between a positive screening test report and confirmation of the positive screening report. So far this year, of the four babies in our catchment area who proved to have classic PKU, initiation of treatment ranged from nine to 15 days of age.

Once the baby arrives in clinic, a set of tests is performed to confirm or reject the diagnosis of PKU. Ideally, the phenylalanine hydroxylase should be measured, but it is normally present only in the liver where it is not readily accessible. A liver biopsy would not be accepted by many parents. However, a series of metabolite tests is in place with which there is long experience. A blood phenylalanine value of 0.15 mM (2.5 mg per dL) separates the phenylketonuria cases from the transient or borderline cases. Those hyperphenylalaninemia disorders owing to a defect in the phenylalanine hydroxylase regularly show a blood phenylalanine greater than 0.24 mM (4 mg per dL) and a blood tyrosine less than 0.11 mM (2 mg per dL). Decisions on what degree of hyperphenylalaninemia to treat varies from clinic to clinic, but the blood phenylalanine level of 0.48
mM (8 mg/dL) is used by us. This leaves only two classes of hyperphenylalaninemia: PKU and benign hyperphenylalaninemia.

In 1975, Kaufman and co-workers reported a child with a new form of PKU, a situation in which the apoenzyme, phenylalanine hydroxylase, was intact, but a coenzyme was absent, leading to BH4 deficiency. Now, three defects leading to BH4 deficiency have been delineated. The BH4 deficient forms of hyperphenylalaninemia must be identified, for they do not respond to the low phenylalanine diet, thus the label of malignant hyperphenylalaninemia. Additional diagnostic methods for identification and classification of these variant forms of hyperphenylalaninemia have been developed. They depend on the amounts and ratios of neopterin and biopterin in the urine for the two synthesis defects and on direct assay of the enzyme in red cells for the reductase defect. Both blood and urine for these tests can be collected on filter paper.

### Case Interpretations

The results indicating which hyperphenylalaninemia disorder is under study requires a battery of tests, but correct interpretation allows the clinician to provide or withhold the low phenylalanine diet with some conviction.

Normal subjects have half or more of biopterin (B) in the tetrahydro forms with a neopterint (N) to biopterin ratio of approximately one. The urine ratio N/B is elevated in the first month of life. Patients with PKU have elevated values for both N and B. However, the N/B ratio and percent BH4 are normal. Dihydropteridine reductase (DHPR) deficient patients have an elevated N/B ratio and total values for N and B but greatly reduced or absent BH4 values. Typical 6-Pyruvoyltetrahydropterin synthase (6-PTS) deficient patients have greatly increased neopterin and N/B ratio.

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### TABLE I

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Specimen</th>
<th>Test</th>
<th>Results Indicating Disorder</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKU, classic (phenylalanine hydroxylase) deficiency</td>
<td>Serum</td>
<td>Quantitative amino acid analysis</td>
<td>Phenylalanine &gt;0.48 mM or 8 mg/dL Tyrosine &lt;0.11 mM or 2 mg/dL Neopterin Increased Bioterin Increased N/B Normal (near 1.0)</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>HPLC of metabolite</td>
<td>Neopterin</td>
</tr>
<tr>
<td>Benign hyperphenylalaninemia (phenylalanine hydroxylase deficiency)</td>
<td>Serum</td>
<td>Quantitative amino acid analysis</td>
<td>Phenylalanine &lt;0.48 mM or 8 mg/dL Tyrosine &lt;0.11 mM or 2 mg/dL Neopterin Increased Bioterin Increased N/B Normal (near 1.0)</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>HPLC of metabolite</td>
<td>Neopterin</td>
</tr>
<tr>
<td>Malignant hyperphenylalaninemia I (dihydropteridine reductase deficiency)</td>
<td>Blood</td>
<td>Enzyme assay</td>
<td>DHPR &lt;1.4 nmol/min/mg Hgb Neopterin Increased Bioterin Increased N/B Low or normal</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>HPLC of metabolite</td>
<td>Neopterin</td>
</tr>
<tr>
<td>Malignant hyperphenylalaninemia II (6-Pyruvoyltetrahydropterin synthase deficiency)</td>
<td>Urine</td>
<td>HPLC of metabolite</td>
<td>Neopterin Increased Bioterin Decreased N/B High</td>
</tr>
<tr>
<td>Malignant hyperphenylalaninemia III (guanosine triphosphate cyclohydrolase deficiency)</td>
<td>Urine</td>
<td>HPLC of metabolite</td>
<td>Biopterin Trace amount Neopterin Trace amount N/B Normal (near 1.0)</td>
</tr>
</tbody>
</table>

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* Degradation product of BH4.
† Degradation product of dihydroneopterine triphosphate, the substrate of 6-PTS.
values. In a subgroup of these patients with only peripheral 6-PTS deficiency, but the cerebral spinal fluid pterin pattern is normal. Guanosine triphosphate cyclohydrolase (GTP-CH) deficient patients have very low total N and B values with a normal N/B ratio.

Phenylalanine metabolism continues to be clarified by experience and study of atypical patients. At this writing, a practical guide for the evaluation and diagnosis of a particular hyperphenylalaninemia patient is shown in table I. Careful clinical and laboratory correlation is required to obtain an accurate diagnosis in the patient with hyperphenylalaninemia. Those that require treatment should commence on the low phenylalanine diet within a few days of birth, and the restriction of their dietary phenylalanine should continue for life.

References


