Blood spots on Guthrie cards can be used for inherited tetrahydrobiopterin deficiency screening in hyperphenylalaninaemic infants

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SUMMARY We describe a method of screening for dihydropteridine reductase deficiency and dihydrobiopterin synthesis deficiency—the two inherited defects that cause tetrahydrobiopterin deficiency—using blood spots on Guthrie cards. Dihydropteridine reductase deficiency may be identified positively, and a biopterin value of <6·0 μg/l in the presence of hyperphenylalaninaemia indicates further investigation for dihydrobiopterin synthesis deficiency.

Tetrahydrobiopterin deficiency can arise from defective synthesis of dihydrobiopterin,1,2 or from lack of dihydropteridine reductase activity,2,3 and is a cause of neonatal hyperphenylalaninaemia. Routine screening for phenylketonuria depends upon detection of raised phenylalanine values but does not distinguish hyperphenylalaninaemia arising from tetrahydrobiopterin deficiency from the far more common cause of hyperphenylalaninaemia, phenylalanine hydroxylase deficiency. Tetrahydrobiopterin is the cofactor for the hydroxylation of tyrosine4 and tryptophan5 as well as phenylalanine. Patients with cofactor deficiency develop gross lack of serotonin, dopamine, and noradrenaline. Phenylalanine restriction alone is not the appropriate treatment for these patients who require additional treatment with L-dopa, carbidopa and 5-hydroxytryptophan.

Dihydropteridine reductase deficiency may be detected by measurement of the enzyme in red blood cells,6,7 by urine pterin analysis,8 or by measurement of total biopterins in plasma.9 Defective dihydrobiopterin synthesis can also be detected by measurement of plasma biopterins2 or urinary pterins.8,10 The liquid specimens for plasma and urine measurements have to be handled with care, protected from light, frozen to −20°C as soon as possible, and transported in solid carbon dioxide.

These techniques can, however, be modified to allow assays of dihydropteridine reductase7 and total biopterin to be carried out on blood eluted from dried spots on Guthrie cards. Narisawa et al.,7 have shown that dried blood spots can be used for assay of reductase activity. Preliminary work in this laboratory has shown that total biopterin is well preserved in dried blood spots, which retain maximal activity for up to four weeks. Use of dried blood spots on Guthrie cards, as an aid to separation of both forms of tetrahydrobiopterin deficiency from phenylalanine hydroxylase deficiency, would simplify and encourage routine testing of all newborn infants with raised phenylalanine concentrations for tetrahydrobiopterin deficiency. Plasma biopterins increase in parallel with phenylalanine in normal subjects and in phenylketonuric children,11,12 but not in dihydrobiopterin synthesis deficiency.2,12 Measurement of whole blood biopterins at diagnosis in infants with tests positive for phenylketonuria (and therefore hyperphenylalaninaemia) should enable those infants with a block in biopterin synthesis to be picked out for further investigation. Patients with dihydropteridine reductase deficiency show a very notable accumulation of biopterin (due to dihydrobiopterin)9 so that biopterin measurements can be used to confirm enzyme results.

We describe the routine use of a Guthrie card technique for measurement of dihydropteridine reductase activity and total biopterins in a series of 44 infants with hyperphenylalaninaemia detected on routine neonatal screening.

Methods

Guthrie cards bearing four blood spots were sent through the post from phenylketonuria clinics and
Inherited tetrahydrobiopterin deficiency screening

screening centres in Britain, France, and Eire. Many of the neonates with positive screening tests for phenylketonuria, had already begun treatment by the time blood was taken for biopterin assay. For comparison with the neonates, 14 older children with phenylketonuria, 19 normal adults, and 9 patients with known tetrahydrobiopterin deficiency were also tested.

When the cards arrived in the laboratory two 8 mm discs were cut into 4-0 ml phosphate buffer pH 5-0, the solution and discs were frozen at −20°C, and later autoclaved for three minutes at 115°C to deproteinise and to elute the biopterin for bioassay with the protozoon Crithidia fasciculata. This assay provides a measure of the combined activity of tetrahydrobiopterin, dihydrobiopterin, biopterin, and sepiapterin. The remaining blood spots were stored at −20°C in sealed plastic bags and were later eluted for dihydropteridine reductase assay. Each incubation contained 0-05 M Tris buffer pH 7-5, 10−5 M NADH, 5 × 10−5 M cytochrome C (Sigma, type III), and 10−5 M 6-methyl 5, 6, 7, 8-tetrahydropteridine with appropriate enzyme solution in a total volume of 1 ml. Plasma phenylalanine results were provided by the centres that generated the samples.

**Results**

Dihydropteridine reductase assays on blood spots from the neonates with hyperphenylalaninaemia gave results similar to those found in normal adults and older patients with phenylketonuria not caused by dihydropteridine reductase deficiency (Table). The neonatal Guthrie card biopterin values correlated closely with phenylalanine concentrations (see Figure). In those infants on a restricted phenylalanine intake, with plasma phenylalanine concentrations within or close to the normal range, biopterin values were similar to those in normal adults and in patients with defective dihydrobiopterin synthesis (see below and Table). The lower limit of the Guthrie card bioassay was 2-4 μg/l and 7 normal subjects, three synthesis deficient patients, and 1 neonate with treated phenylketonuria had values below this. Older children with phenylketonuria also showed biopterin values that correlated closely with those of phenylalanine.

No neonates with suspected tetrahydrobiopterin deficiency were examined during the course of the study. Examination of Guthrie cards from three patients of various ages known to have dihydropteridine reductase deficiency showed that they had

**Table**  Guthrie card biopterins, phenylalanine, and dihydropteridine reductase activity values in normal adults, children with phenylalanine hydroxylase deficiency, dihydrobiopterin synthesis deficiency, and dihydropteridine reductase deficiency

<table>
<thead>
<tr>
<th>Group</th>
<th>Guthrie card biopterin (μg/l whole blood)*</th>
<th>Phenylalanine (μmol/l)</th>
<th>Dihydropteridine reductase activity (nmol NADH/min/l whole blood)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neonates with positive screening test (n=44) (Mean SD))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine &lt;240 μmol/l</td>
<td>3-9 (1-9)</td>
<td>109 (56)</td>
<td>0-157 (0-063)</td>
</tr>
<tr>
<td>Phenylalanine &gt;240 μmol/l</td>
<td>12-8 (7-3)</td>
<td>797 (707)</td>
<td></td>
</tr>
<tr>
<td>Reductase deficient children (n=3) (Range)</td>
<td>21-3-50-3</td>
<td>260-800</td>
<td>0-0-005</td>
</tr>
<tr>
<td>Synthesis deficient children (complete) (n=2) (Range/single value)</td>
<td>2-4-4-8 (2)</td>
<td>—</td>
<td>0-136 (1)</td>
</tr>
<tr>
<td>Synthesis deficient children (partial) (n=4) (Range)</td>
<td>2-4-3-6 (4)</td>
<td>230-890 (2)</td>
<td></td>
</tr>
<tr>
<td>Children aged 4-15 yrs with phenylketonuria (n=14) (Mean SD))</td>
<td>6-45 (1-98)</td>
<td>338 (153)</td>
<td>0-115 (0-042)</td>
</tr>
<tr>
<td>Normal adults (n=19) (Mean SD))</td>
<td>4-67 (2-7)</td>
<td>63 (25)</td>
<td>0-138 (0-370)</td>
</tr>
<tr>
<td>Adult heterozygotes for reductase deficiency (n=3) (Mean SD))</td>
<td>—</td>
<td>—</td>
<td>0-051 (0-060)</td>
</tr>
</tbody>
</table>

*Calculated for comparison with other published results.

1Includes ‘classic’ and ‘atypical’ patients some of whom had started a low phenylalanine diet before the test was done. Italicised figures in parentheses are number of subjects for whom values were available.
The results of the other hand enable them reductase
tions hyperphenylalaninaemia, provided
deficiency occurring
synthesis and those
between patients
screen spots when
value patients
biopterin ketonuria
activity. The enzyme assay
and tions normal.
neat
near
of normal adults and those
measurements on
phenylalanine and
biopterin in
[lmol/l]
gimol/l
phenylalanine hydroxylase
deficiency.

Figure Correlation
Leeming, RJ, Aicardi J, Rey J. Biopterin derivatives in atypical

References
1 Leeming RJ, Blair JA, Rey F. Biopterin derivatives in atypical
2 Rey F, Harpey JP, Leeming RJ, Blair JA, Aicardi J, Rey J. Les
hyperphenylalaninemies avec activite normale de la
dehydroprioterin deficiency is it most important to ensure that the
Guthrie card specimens are obtained while the
patient is on a normal protein intake when phenylalanine
values are maximal. When phenylalanine
values are below 600 µmol/l, either because a low
phenylalanine diet has been introduced or because a
partial block is present, biopterin values in patients
with hydroxylase deficiency overlap with those in
patients with a defect of dihydrobiopterin synthesis.

We suggest that any neonate with a Guthrie
card biopterin value below 6 µg/l should be investigated
further for the possible presence of tetrahydrobiopterin
deficiency. This is probably best done by a
combination of an oral load of tetrahydrobiopterin
(7.5 mg/kg with serial measurements of phenylala-
nine and tyrosine) and measurement of either urine
pterins or plasma biopterins. A phenylalanine load
may also be used to show a defective biopterin
synthesis but should be used cautiously because
symptoms may be precipitated by this procedure.

The progressive neurological symptoms of inher-
ited tetrahydrobiopterin deficiency may be arrested
by giving the neurotransmitter precursors l-dopa
and 5-hydroxytryptophan, or prevented if treat-
ment is started in the neonatal period. The pro-
gnosis without appropriate treatment is generally
poor, with profound neurological damage, gross
hypotonia, and death in early childhood, usually
from recurrent respiratory tract infection. The
incidence of the disease is low (approximately
1/600 000 births) but the percentage of patients with
phenylketonuria who may have this variant has been
calculated at between 1% and 3%. In the United
Kingdom between 150 and 200 infants each year
have raised phenylalanine values (over 240 µmol/l)
persisting beyond the time of the first repeat of the
positive routine screening test. All these infants
should be tested for tetrahydrobiopterin deficiency
even if phenylalanine values are not high enough to
require treatment with a low phenylalanine diet.

The screening technique described here must
commend itself to paediatricians and parents alike
by its convenience and simplicity. Further experi-
ence of the test on much larger numbers of infants
on a normal diet will enable the technique to be
further evaluated, including any false positive or
false negative results.

We are indebted to the directors of screening laboratories who
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Inherited tetrahydrobiopterin deficiency screening


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