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 $<6.0 \mu\text{g/l}$ 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 blood 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

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 protozoan *Crithidia fasciculata*.¹³ 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^{-4} M NADH, 5×10^{-5} M cytochrome C (Sigma, type III), and 10^{-5} M 6-methyl 5, 6, 7, 8-tetrahydropterin 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 $2.4 \mu\text{g/l}$ 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

Group	Guthrie card biopterin ($\mu\text{g/l}$ whole blood)*	Phenylalanine ($\mu\text{mol/l}$)	Dihydropteridine reductase activity (mmol NADH/min/l whole blood)*
Neonates with positive screening test [†] (<i>n</i> =44) (Mean (SD))			
Phenylalanine <240 $\mu\text{mol/l}$	3.9 (1.9)	109 (56)	0.157 (0.063) (<i>n</i> =39)
Phenylalanine > 240 $\mu\text{mol/l}$	12.8 (7.3)	797 (707)	
Reductase deficient children (<i>n</i> =3) (Range)	21.3–50.3	260–800	0–0.005
Synthesis deficient children (complete) (<i>n</i> =2) (Range/single value)	2.4–4.8 (2)	—	0.136 (1)
Synthesis deficient children (partial) (<i>n</i> =4) (Range)	2.4–3.6 (4)	230–890 (2)	—
Children aged 4–15 yrs with phenylketonuria (<i>n</i> =14) (Mean (SD))	6.45 (1.98)	338 (153)	0.115 (0.042)
Normal adults (<i>n</i> =19) (Mean (SD))	4.67 (2.7)	63 (25) (14)	0.138 (0.370) (16)
Adult heterozygotes for reductase deficiency (<i>n</i> =3) (Mean (SD))	—	—	0.051 (0.060)

*Calculated for comparison with other published results.

[†]Includes '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.

Figure Correlation between Guthrie card bipterins and phenylalanine values in 44 neonates with phenylalanine hydroxylase deficiency.

even more notably raised blood bipterin concentrations than patients with phenylalanine hydroxylase deficiency, and very low dihydropteridine reductase activity. The enzyme assay distinguished obligate heterozygotes (parents) from normal.

Six patients with disorders of bipterin synthesis (complete in two and partial in four) had Guthrie card bipterin results indistinguishable from those of normal adults and those found in the neonates whose phenylalanine concentrations were normal or near normal. These results contrasted with plasma measurements on the same patients that gave low bipterin values. None of the neonates with phenylketonuria who had phenylalanine values above 600 $\mu\text{mol/l}$ had Guthrie card bipterin values below 6.0 $\mu\text{g/l}$ (see Figure), whereas the highest bipterin value on a patient with dihydrobiopterin synthesis deficiency was 4.8 $\mu\text{g/l}$. Clearly, separation between patients with dihydrobiopterin synthesis deficiency and phenylalanine hydroxylase deficiency is best when phenylalanine values are high.

Discussion

The results of the present study suggest that dried blood spots from Guthrie cards can be used to screen for the two types of tetrahydrobiopterin deficiency occurring among infants with neonatal hyperphenylalaninaemia, provided that the limitations of the method are recognised. Patients with dihydropteridine reductase deficiency have results that enable them to be easily identified. On the other hand to achieve reasonable separation between patients with defects of dihydrobiopterin synthesis and those with phenylalanine hydroxylase

deficiency it is 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 $\mu\text{mol/l}$, either because a low phenylalanine diet has been introduced or because a partial block is present, bipterin 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 bipterin value below 6 $\mu\text{g/l}$ should be investigated further for the possible presence of tetrahydrobiopterin deficiency. This is probably best done by a combination of an oral load of tetrahydrobiopterin¹⁴ (7.5 mg/kg with serial measurements of phenylalanine and tyrosine) and measurement of either urine pterins⁸ or plasma bipterins.² A phenylalanine load may also be used to show a defective bipterin synthesis¹¹ but should be used cautiously because symptoms may be precipitated by this procedure.¹⁵

The progressive neurological symptoms of inherited tetrahydrobiopterin deficiency may be arrested by giving the neurotransmitter precursors L-dopa and 5-hydroxytryptophan,¹⁶ or prevented if treatment is started in the neonatal period.⁹ The prognosis without appropriate treatment is generally poor, with profound neurological damage, gross hypotonia, and death in early childhood, usually from recurrent respiratory tract infection.¹⁷ 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 $\mu\text{mol/l}$) 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 experience 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.

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