J. Inherit. Metab. Dis. 28 (2005) 651–663
DOI: 10.1007/s10545-005-0033-x

Benzoate treatment and the glycine index in nonketotic hyperglycinaemia

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MS received 23.09.04 Accepted 02.02.05

Summary: High-dose benzoate treatment aimed at reducing plasma glycine levels to normal reduces seizures and increases wakefulness in patients with nonketotic hyperglycinaemia (NKH). Since benzoate metabolism is dependent on the available glycine pool, and since the glycine pool is variably affected by the deficiency in the glycine cleavage enzyme system, we examined the importance of interpatient variability in benzoate requirement. To correct for the dietary glycine contribution, the glycine index was introduced as the molar requirement of benzoate dose necessary to normalize plasma glycine levels and subtracting from that the dietary glycine intake, both corrected for weight. The glycine index varied between 3.62 and 4.87 mmol/kg per day in five patients with a poor neurodevelopmental outcome and between 0.92 and 1.90 mmol/kg per day in four patients with a better neurodevelopmental outcome, and was 2.54 mmol/kg per day in a single patient with an intermediate outcome. The glycine index was stable over time within each patient. Exceeding the balance by either increasing food glycine intake or decreasing the benzoate dose resulted in increased glycine levels. Exceeding the glycine tolerance by increasing benzoate resulted in elevated and toxic levels of benzoate. The glycine index is a stable, individually specific parameter in patients with NKH. It has clinical consequences for the dose of benzoate required and the role of dietary management. Through its correlation with neurodevelopmental outcome, the glycine index points to potential genetic factors that could contribute to the psychomotor retardation in NKH.

Nonketotic hyperglycinaemia (McKusick 606899) is an autosomal recessive condition caused by deficient enzyme activity of the glycine cleavage enzyme system (EC 2.1.1.10) (Hamosh and Johnston 2001). The glycine cleavage enzyme system comprises four proteins: P-, T-, H- and L-proteins (EC 1.4.4.2, EC 2.1.2.10 and EC 1.8.1.4 for P-, T- and L-proteins). Mutations have been described in the *GLDC* (McKusick 238300), *AMT* (McKusick 238310), and *GCSH* (McKusick 238330) genes encoding the P-, T-, and H-proteins respectively. The glycine cleavage system catalyses the oxidative conversion of glycine into carbon dioxide and ammonia, with the remaining one-carbon unit transferred to folate as methylenetetrahydrofolate. It is the main catabolic pathway for glycine and it also contributes to one-carbon metabolism. Patients with a deficiency of this enzyme system have increased glycine in plasma, urine and cerebrospinal fluid (CSF) with an increased CSF:plasma glycine ratio.

Clinically, most patients present in the neonatal period with lethargy, coma, seizures and apnoea. Apnoea improves spontaneously after 2 weeks, but most patients develop severe mental retardation and severe seizures. Congenital brain malformations such as agenesis or hypoplasia of the corpus callosum, and the combination of a retrocerebellar cyst with hydrocephalus have been described (Dobyns 1989; Van Hove et al 2000). Patients with late-onset variant forms exhibit variable degrees of mental retardation and seizures (Hamosh and Johnston 2001; Steiner et al 1996). Rare patients have been described with the features of neonatal NKH, but whose biochemical and clinical symptoms disappeared in infancy. The outcome of these patients has been variable (Aiefendioğlu et al 2003). The aetiology of this transient form is unknown at present.

There is currently no effective treatment for NKH. Most patients are treated with benzoate and dextromethorphan (Hoover-Fong et al 2004). Benzoate, after activation to its coenzyme A ester, conjugates with glycine to form hippurate, which is efficiently excreted in the urine. Providing high doses of benzoate allows for the removal of large amounts of glycine in the urine, and results in a reduction of plasma glycine levels to normal. This treatment reduces seizures and improves alertness; however, it does not prevent the development of mental retardation (Hamosh et al 1998; Van Hove et al 1995; Wolff et al 1986). The dose of benzoate used has ranged from 250 to 750 mg/kg per day. Doses exceeding 750 mg/kg per day are associated with toxicity. Plasma benzoate levels must be monitored to prevent toxicity (Van Hove et al 1995). Dextromethorphan blocks the NMDA receptor, for which glycine is an allosteric activator (Hamosh et al 1998; Deutsch et al 1998).

In this study we explored the dose of benzoate required to normalize the plasma glycine level in relation to the food glycine intake in patients with NKH, conceptually defined as the glycine index.

METHODS

Ten patients with NKH, 7 male and 3 female, ranging in age from 1 month to 13 years, were studied (Table 1). The diagnosis was based on elevated glycine in plasma and CSF with an elevated CSF:plasma glycine ratio greater than 0.08. All patients had a normal urine organic acids analysis. The diagnosis was confirmed by deficient enzyme activity in liver in 4 patients and in lymphoblasts in 3 patients and mutations were identified in the *GLDC* or the *AMT* gene in 9 patients (details published elsewhere). A developmental quotient was made at age >2 years by standardized assessment, in most cases the Bailey

Table 1 Glycine index in patients with nonketotic hyperglycinaemia

Patient no.	*Sex*	*Onset*	*Severity*	*Malformation*	*Psychomotor development*	*Antiepileptic drugs*	*Glycine index*	*Gly + Ser index*
1	F	1st week	Severe	Cerebellar cyst	<6 weeks	4	4.66	3.99
2	M	1st week	Severe	Cerebellar cyst	<6 weeks	3	3.88	3.46
3	M	2 months	Severe	Hypoplastic CC	<6 weeks	4	3.83	3.07
4	M	1st week	Severe	None	<6 weeks	3	3.62	2.92
5	M	6 weeks	Severe	None	<6 weeks	3	4.81	4.11
6	M	1st week	Intermediate	None	8 mo, DQ 6	0	2.54	1.58
7	F	1st week	Moderate	None	DQ 23	0	0.92	−0.39
8	M	1st week	Moderate	None	DQ 21	1	1.90	1.90
9	M	1st week	Mild	None	DQ 30	0	1.80	1.54
10	F	1 year	Mild	None	DQ 55	0	1.51	0.38

Glycine index and glycine-serine index in mmol/kg per day were calculated in 10 patients with NKH Development: maximal developmental age; or DQ, developmental quotient
Severity is based on both neurodevelopmental outcome and severity of the seizure disorder reflected in the number of antiepileptic medications
M, male; F, female; hypoplastic CC, hypoplastic corpus callosum

Scales of Mental Development. The presence of brain malformations was documented by magnetic resonance imaging. Clinical studies were approved by the Ethics Committee of the University Hospital Gasthuisberg Leuven, and informed consent was obtained.

As part of the therapy for NKH, patients received increasing doses of sodium benzoate until plasma glycine was in the low-normal range (100–260 μmol/L). Sodium benzoate was usually administered orally or by gastric tube as a 10% solution in water divided in 3–6 daily doses. If enteral administration was not possible, sodium benzoate was administered continuously as a 10% intravenous solution. Plasma glycine and benzoate levels were obtained 1–2 h after the second dose of the day. In some patients, as part of adjusting medical care, several glycine and benzoate levels were obtained throughout the day, providing information about the diurnal variation of glycine and benzoate levels.

Plasma amino acids were measured as their ninhydrin derivatives on a Biochrom amino acid analyser. Plasma benzoate levels were assayed by reversed-phase HPLC using a modification of a previously published method (Batshaw et al 1982; C. Bachmann, personal communication, 1998).

The intake of glycine and serine from food was calculated from 3-day dietary records and from observations in the hospital. To calculate the glycine and serine contents of foods, tables of glycine and serine contents of common food products were compiled from standard European textbooks of food content (Souci et al 2000) and by contacting manufacturers (Schoovaerts 2001). For all infant formulas, tube feedings, and metabolic formulas available in Belgium and Germany, this information was obtained from the manufacturers.

The glycine index is a new parameter developed to study whole-body glycine metabolism in NKH patients. It is defined as follows. Theoretically, 1 mole of benzoate removes 1 mole of glycine as hippurate. In NKH patients, at least 75% of ingested benzoate is excreted as hippurate (Barshop et al 1989), and hippurate excretion is linearly related to the benzoate dose. The benzoate dose thus reflects the hippurate excretion. After adjusting the benzoate

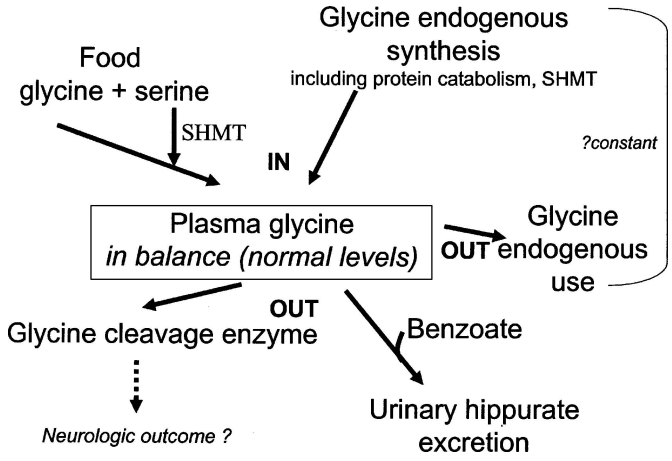

Figure 1 The glycine index. If the plasma glycine is stable in the normal range, the glycine index can be derived from the difference between the benzoate dose required, as an estimate of the urine hippurate excretion, and the food intake of glycine (± serine). SHMT, serine hydroxymethyltransferases. Various metabolic routes lead to the synthesis of glycine, including the serine hydroxymethyltransferase and protein catabolism (here called glycine endogenous synthesis). Other metabolic routes lead to the use and hence disappearance of glycine such as oxidation to glyoxylate; use in synthesis of proteins, purines, creatine, porphyrins and glutathione; and use in conjugation with bile acids (here called 'glycine endogenous use'). It is assumed that the difference between the glycine endogenous synthesis and the glycine endogenous use, if expressed in relation to weight, is constant, at least in children over 1 year of age

dose to normalize plasma glycine levels (target range 100–260 μmol/L), in steady state, the glycine index was calculated as the difference between the molar dose of benzoate reflecting the amount of glycine excreted in urine as hippurate, and the amount of glycine ingested from food. Serine can be converted into glycine through the serine hydroxymethyltransferase enzymes, although its contribution in NKH to the glycine pool is not known at present. Therefore, both the glycine index and the combined glycine-serine index were calculated.

The glycine index therefore reflects the net endogenous synthesis of glycine minus the residual glycine cleavage enzyme activity, hence the endogenous glycine excess. Theoretically, if the sum of the glycine synthesis and glycine catabolism by other pathways is constant for weight, then the glycine index reflects whole-body residual activity of the glycine cleavage reaction, which could be related to the severity of the NKH (Figure 1). Alternatively, this may also reflect polymorphisms at other glycine synthetic or catabolic pathways that could potentially modify the prognostic outcome. In practice, the glycine index is calculated as the dose of benzoate required on a molar basis, minus the glycine or the glycine + serine intake from diet on a molar basis, expressed on a per weight basis. For example:

Glycine index (mmol/kg per day)
= [benzoate dose (mmol) − glycine food (mmol)]/weight (kg)
Glycine-serine index (mmol/kg per day) = [benzoate dose (mmol)
− (glycine food (mmol) + serine food (mmol))]/weight (kg)

To assess the internal validity of the glycine index (glycine-serine index), the index was calculated repeatedly in 3 patients over several months.

RESULTS

For the practical assessment of glycine levels, it was important to know how levels varied throughout the day in NKH patients on high-dose benzoate during a typical home schedule. Serial plasma glycine and benzoate levels were obtained in a patient with classical NKH given 750 mg/kg per day sodium benzoate divided in 3 doses (Figure 2). A stable plasma glycine level was achieved 2 h after one dose of benzoate. However, during the 12 h interval of the night, benzoate levels dropped below the measurable range (<0.1 mmol/L) and glycine levels rose above the treatment range. It is of note that in this and subsequent patients, such elevation of glycine during the night was not associated with clinical symptoms until benzoate had been withheld for more than 18 h. In subsequent patients, levels were obtained at least 2 h after the morning benzoate dose. Patients with severe NKH with plasma glycine levels in the target range usually had peak plasma benzoate levels less than 2.5 mmol/L.

A compilation of the percentage contribution of glycine and serine to the total protein showed substantial variation between foods (Table 2). Milk products, including milk-based infant formulas, naturally rich in essential amino acids, had a lower glycine + serine content than soy-based infant formulas or a meat protein hydrolysate-based infant formula. Most childhood tube feeding formulas are also cow's milk protein-based and had a similar low glycine + serine content. Eggs, wheat and flour, fish and meats have a higher glycine

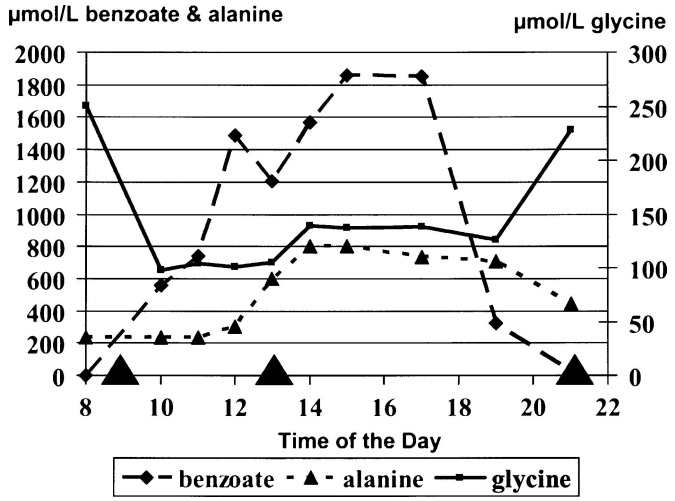

Figure 2 Diurnal variation of glycine and benzoate levels during treatment. The plasma levels of benzoate, glycine and alanine vary throughout the day in a patient receiving benzoate at 750 mg/kg per day in 3 divided doses. The administration time of benzoate is indicated by triangles on the *x*-axis. Solid line, glycine level, dashed line, benzoate level, dotted line, alanine level

Table 2 Glycine + serine content of food

Food category	*Glycine + serine* (mg/g protein)	*Comments*
Milk products, including milk-based infant formulas and cheeses	60–86	Lowest: Pepti-Junior® (Nutricia) (61)
Soy-based infant formulas	93–96	
Meat protein-based infant formula (Pregomin® Milupa)	140	
Childhood tube feeding	61–86	Lowest: Nutrison Pepti® (Nutricia) (61)
Eggs	114–140	
Grains and flour	108–130	High: millet and buckwheat
Fish and meats	117–123	Low: lamb (92) High: hake, catfish, mackerel
Organ meat	133–146	
Gelatin	314	
Vegetables and fruits	43–148	High (>100) potatoes, soy, beans Low (<70) tomatoes, carrots, green leaf vegetables, mushrooms

+ serine contribution. Gelatin, mainly composed of collagen, has extremely high glycine content. This can be of practical importance since capsules are often made of gelatin. This contributed more than 30% of glycine intake in one patient taking benzoate in such capsules. Vegetables and fruits had very variable glycine + serine content. Fruits had low total protein content, thus limiting the glycine + serine content.

The intake of glycine and serine from food by the patients varied widely. The glycine intake varied from 270 mg/day (3.6 mmol/day) to 1658 mg/day (22.1 mmol/day), and from 23 mg/kg per day (0.31 mmol/kg per day) to 106 mg/kg per day (1.42 mmol/kg per day). The serine intake varied from 442 mg/day (4.2 mmol/day) to 1987 mg/day (18.9 mmol/day), and from 27 mg/kg per day (0.26 mmol/kg per day) to 138 mg/kg per day (1.31 mmol/kg per day).

The glycine index was calculated in 10 patients (see Table 1). A glycine index of more than 3 mmol/kg per day (range 3.62–4.58) and a glycine-serine index more than 2.5 mmol/kg per day (range 2.92–4.11) was found in 5 patients. These patients had severe NKH. Development was very poor, with no new milestones achieved; none of the patients learned to sit or grasp. All of them had a developmental age of less than 6 weeks. They each had severe epilepsy requiring three or more anticonvulsants. Two patients had a retrocerebellar cyst with hydrocephalus, one had hypoplasia of the corpus callosum. A glycine index lower than 2 mmol/kg per day (range 0.92–1.90) and a glycine-serine index lower than 2 mmol/kg per day (range −0.39 to 1.90) was found in patients with a better developmental outcome. These patients made developmental progress. They learned to sit, grasp objects, and communicate with their caregivers. The epilepsy was mild and was controlled with benzoate alone or with the addition of a single antiepileptic medication such as dextromethorphan. One patient with an intermediate glycine index of 2.54 mmol/kg per day and a glycine-serine index of 1.58 mmol/kg per day had an intermediate prognosis: he learned to sit, but he could not

Table 3 Consistency of the glycine index

			Glycine index		*Glycine-serine index*	
Patient	*N*	*Time interval*	Mean ± SD	CV %	Mean ± SD	CV %
1	7	24 months	4.66 ± 0.30	6.5	3.99 ± 0.70	17.6
2	3	13 months	3.88 ± 0.35	9.1	3.46 ± 0.42	12.1
3	3	1 month	3.83 ± 0.16	4.3	3.07 ± 0.12	4.0
6	6	16 months	2.54 ± 0.22	8.6	1.58 ± 0.19	12.0

Reproducibility of the glycine index and of the glycine-serine index in 4 patients calculated over a period ranging from 1 to 24 months. *N*, number of balances calculated; CV, coefficient of variation

purposefully grasp and had little social interaction. The difference in glycine index between both classes of NKH patients, those who made developmental progress and those who did not, was statistically significant (t-test, $p < 0.01$).

To establish the reproducibility, the balance was calculated repeatedly in 3 patients (Table 3). The glycine index was stable over time in these patients more than 1 year of age with an average coefficient of variation of 7.1%. The average coefficient of variation for the glycine-serine index was 11.4%. At present, we do not have adequate data in patients less than 1 year of age.

The differences in glycine index have therapeutic implications. It is unclear to what extent serine contributes and, for practical reasons, the subsequent calculations will only include the glycine index. The difference in glycine index between less severely and more severely affected patients has obvious implications for the dose of benzoate required to maintain glycine in the target range of 100–260 μmol/L. In patients with severe NKH and a high glycine index, varying between 3.62 and 4.81 mmol/kg per day, the benzoate dose required is 522–693 mg/kg per day, not taking into account the glycine intake from food. The use of benzoate doses higher than 750 mg/kg per day has been associated with an often fatal syndrome of renal dysfunction, haematuria and hypocalcaemia (Wolff et al 1986). This high benzoate requirement has implications for the allowed dietary intake of glycine. Let us apply this to a hypothetical patient with severe NKH with an average weight of 10 kg. Based on the above data and given the maximum tolerable dose of benzoate of 750 mg/kg per day, a food glycine intake of less than 27–118 mg/kg per day is allowed. Most of these patients are tube fed. One litre of a cow's milk-based standard infant formula, which contains the lowest amount of glycine, at 0.67 kcal/ml providing a marginal caloric intake of 67 kcal/kg per day, would already provide on average 409 mg glycine. However, 1 L of a soy-based infant formula at 0.67 kcal/mL would provide 831 mg glycine, and 1 L of standard child tube feeding at 1.0 kcal/ml would provide 550 mg glycine for Nutrison Pediatric Standard (Nutricia) and 540 mg for Pediasure (Abbott). Both the soy formula and the tube feeding would exceed the glycine limitation necessary to maintain the glycine index in the most severely affected patient. This is even more important if solid food is introduced, since solids, whether containing grains or meats, have a much higher content of glycine and serine per gram of protein and per calorie. Therefore, in patients with a high glycine index, restriction of dietary glycine (and possibly serine) is important. Milder patients with a glycine index of 0.92–1.90 mmol/kg per day require benzoate doses of only 133–274 mg/kg

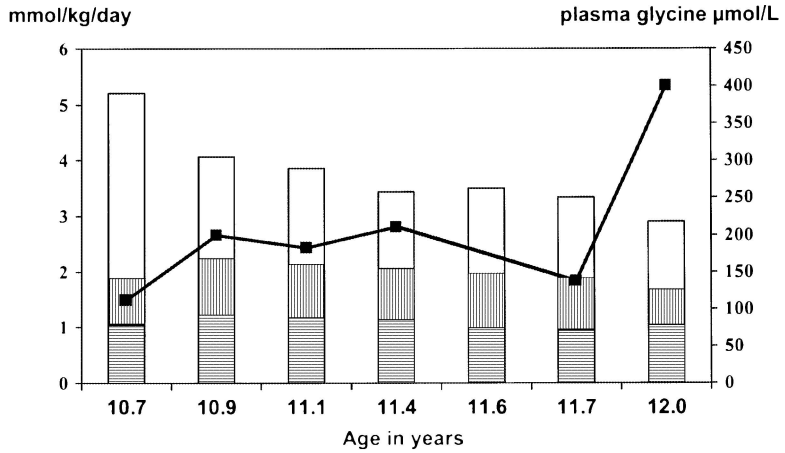

Figure 3 Reduction in benzoate dose. The effect over time on plasma glycine concentrations of the gradual decrease in benzoate dosing in patient 6 is shown. Horizontally hatched bar, glycine intake; vertically hatched bar, serine intake; total bar height, benzoate intake; each in mmol/kg per day. The glycine + serine index is the diiference between the total bar and the hatched bars, i.e. the non-hatched bar height. Plasma glycine concentrations (solid line) increase as the glycine + serine index drops below a threshold at age 12 years

per day. To cover the observed food glycine intake range of 0.33–1.42 mmol/kg per day, the additional benzoate dose required varies between 48 and 205 mg/kg per day. Thus, the total dose of benzoate required can vary between 180 and 480 mg/kg per day, depending on both tolerance and food composition.

Monitoring the glycine index, including the dietary contribution, is important in patients with severe NKH. Indeed, glycine levels exceeded the normal range when the benzoate dose was decreased over time (Figure 3), and when the food glycine content increased without adjustment of the benzoate dose (Figure 4). Such elevations of glycine levels were frequently associated with increased seizures, particularly in the severely affected patients. In the latter case, the plasma glycine levels were normalized by reducing glycine intake without changing the benzoate dose. While maintaining a constant sodium benzoate dose of 725 mg/kg per day, the intake from food was reduced from 0.61 to 0.26 mmol/kg per day glycine and from 1.44 to 0.62 mmol/kg per day serine. The excretion of free glycine and serine in urine fell within 12 h from 0.17 and 0.0048 mmol/kg per day to 0.027 and 0.00073 mmol/kg per day, respectively, and remained in that range in the next days. The plasma glycine level returned to the target range in 1 week.

Finally, increasing the benzoate dose above that required for the glycine index carries the risk of benzoate toxicity. One patient was inadvertently administered the same volume of benzoate as a 25% solution instead of a 10% solution. In 18 h after four administrations, the patient was in coma, vomited and aspirated, had metabolic acidosis and Kussmaul breathing requiring intubation and ventilation. She had severe hypernatraemia (154.4 mmol/L), hypokalaemia (2.16 mmol/L), large anion gap 32.8 mmol/L, hypocalcaemia (6.11 mg/dl; normal values 8.80–10.80), and mild hyperammonaemia 75 µmol/L (normal 11–32).

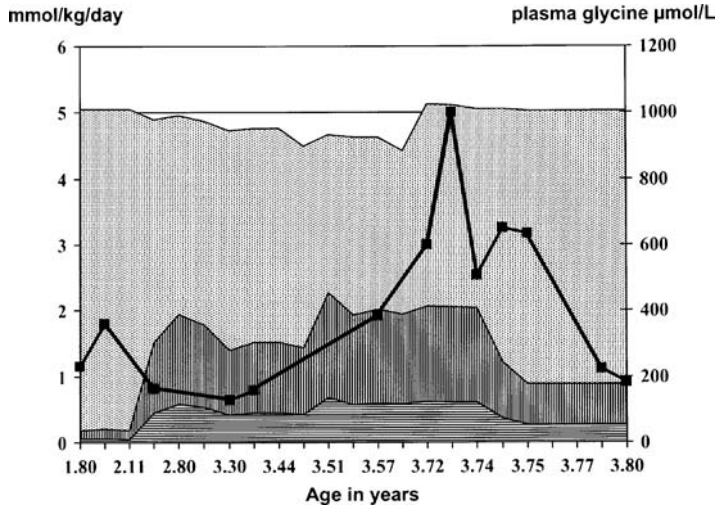

Figure 4 Increased glycine intake. Between ages 3.5 and 3.7 years, while increasing food intake because of failure to thrive, the glycine and serine intake had increased in patient 1. Owing to weight gain, benzoate dosing had slightly decreased. When plasma levels of glycine increased above the target range between age 3.5 and 3.7 years, first the benzoate dose was increased without effect on plasma glycine levels. Then the glycine + serine intake from food was decreased, resulting in lower plasma glycine levels. Horizontal hatching, glycine intake; vertical hatching, serine intake; total height, benzoate intake; each in mmol/kg per day. The glycine + serine index is the difference between the total height and the height of the hatched area, i.e. the height of the dotted area. Plasma glycine levels (solid line) increase as the glycine + serine index drops (from 3.57 years on); when the glycine + serine content in the food is decreased (from 3.75 years on), plasma glycine levels decrease again

Transaminases were normal. Glycine was 62 µmol/L (control 100–350). The benzoate level was 15.4 mmol/L. She was stabilized by discontinuation of benzoate, providing additional glycine, potassium and calcium. She received conventional haemodialysis through a femoral line for 3 h, except using a higher sodium concentration (150 mEq/L) because of the hypernatraemia. Immediately after the dialysis, the benzoate level dropped to less than 0.1 mmol/L. The next morning the patient recovered and previous therapy was reinstituted.

DISCUSSION

After activation to its coenzyme A ester, benzoate is conjugated primarily with glycine. The limiting factor to benzoate metabolism upon increasing doses of benzoate is the availability of the cofactors—glycine for conjugation and coenzyme A for activation—rather than the enzyme activity (Gregus et al 1992, 1993). Owing to the decreased glycine catabolism, the glycine pool available for conjugation with benzoate is substantially greater in NKH than in other conditions such as urea cycle defects. It is therefore to be expected that doses of benzoate needed for reducing plasma glycine levels to normal can be substantially higher in NKH patients than in patients with urea cycle defects (Barshop et al 1989; Wolff

et al 1986). Initial attempts at reducing glycine levels in NKH with doses of benzoate of 250 mg/kg per day, similar to those in urea cycle disorders patients, did not result in a substantial reduction of glycine levels or in improvement of symptoms (Gitzelmann and Steinmann 1982; Krieger et al 1977). Increasing the benzoate dose to 750 mg/kg per day lowered plasma glycine levels and improved patients' symptoms (Wolff et al 1986). The glycine used for conjugation with benzoate in the liver is derived by liver synthesis and by transport from other organs (Qureshi et al 1986). Factors that theoretically can influence the extent of the glycine pool include variations in pathways involved in the synthesis and catabolism of glycine, including the serine hydroxymethyltransferases, residual activity of the glycine cleavage enzyme system, and the amount of glycine in food. The endogenous component of the glycine pool is reflected in the glycine index. Our study demonstrates that the glycine index is a stable parameter in an individual patient, but that it differs substantially between patients.

This individual variability in the required dose of benzoate has many consequences. When the dose of benzoate exceeds the availability of glycine, and glycine levels are below normal, benzoate has only very limited alternative possibilities for conjugation and excretion, such as the formation of benzoylalanine and benzoylglucuronide (Bridges et al 1970; Shinka et al 1985). Renal excretion of unconjugated benzoate is minimal (Barshop et al 1989). This results in an exponential increase in plasma benzoate levels with increasing benzoate dose. Because the aim of the benzoate treat ment of NKH is the reduction of glycine levels to low normal, at which symptomatic improvement occurs, treated patients have a limited glycine pool, and plasma benzoate levels have already risen to 0.5–2.5 mmol/L. Minor increases in the dose above this therapeutic level result in rapidly rising benzoate levels, which result in benzoate toxicity as outlined in the last case. In healthy adult volunteers, symptoms of toxicity were frequent at peak plasma levels of 3.4 mmol/L, but absent at peak levels of 2.1 mmol/L (MacArthur et al 2004). Levels over 3.0 mmol/L should preferably be avoided. Thus, benzoate treatment in NKH has a narrow, patient-dependent, therapeutic window. To prevent benzoate toxicity, we gradually increase the dose of benzoate, e.g. by 50 mg/kg per day, and follow plasma glycine and benzoate levels before any subsequent dose change. This study now provides a guideline for a starting point: in mild patients benzoate therapy can start at 200 mg/kg per day, whereas in severe patients benzoate therapy can be initiated at 500 mg/kg per day.

In normal persons and in patients with urea cycle disorders, benzoate has a short half-life, plasma benzoate levels usually returning to normal in less than 10 h (MacArthur et al 2004). Serial levels of glycine and benzoate measured throughout the day exhibit similar variations in glycine levels in NKH patients despite the higher dose used. Based on the experience as illustrated in Figure 1 and repeated, although less extensively, in other patients, we recommend obtaining glycine and benzoate levels at least 2 h after the first dose, but preferably in the afternoon. In our experience, plasma levels of glycine between 300 and 350 μmol/L, although within the normal range, may well reflect inadequate control. Other levels obtained at the same dose throughout the day frequently exceed the normal values. In patients who do not receive benzoate during the night, benzoate levels have dropped to below 0.1 mmol/L after 12 h sleep, and glycine levels have increased above the normal range. Fortunately, in patients older than 1 year of age, there seems to be a delay in the reappearance of symptoms for several hours beyond this, allowing a practical regimen with

uninterrupted natural sleep. When monitoring plasma levels of glycine and benzoate, this should be taken into account.

Diet alone has been reported to be without effect in NKH (Trijbels et al 1974; reviewed in Gitzelmann and Steinmann 1982), and thus has been abandoned. Our study illustrates that the proper place for a glycine-restricted diet is to assist in maintaining glycine index in severely affected patients who require high doses of benzoate. The story of patient 1 (Figure 4) illustrates how a reduction of glycine intake can assist in achieving normal plasma glycine levels. Reducing the glycine intake will allow for a lower dose of benzoate, limiting side-effects such as gastric irritation, a dose-dependent side-effect. The use of benzoate in excess of 750 mg/kg per day has caused other side-effects, including renal tubular dysfunction, glycosuria, hypokalaemia and hypocalcaemia, and can be fatal (Wolff et al 1986; Van Hove, unpublished observation). Thus, 750 mg/kg per day constitutes a ceiling dose for the safe use of benzoate in NKH patients. In patients who have the highest glycine index, restriction of glycine intake is required to maintain glycine index without exceeding the dose of 750 mg/kg per day. In contrast, for patients with a low glycine index, diet is not necessary. For the most severely affected patients, a specific glycine-free formula would be an advantage but is currently not commercially available. Milk-derived formulas, including tube feedings, have a low percentage of glycine of the total protein. Caution should be used when switching from milk-based formula to soy-based formula or when introducing solids containing grain or meat, which have a substantially higher glycine + serine content.

The contribution of dietary serine to the glycine index is unknown but has practical consequences for the development of a diet in NKH. In experiments using normal mice hepatocytes, serine as a precursor can partially substitute for glycine in the benzoate conjugation (Qureshi et al 1989). In our study, the glycine-serine index had a greater intra-patient variability over time than the glycine index; and the glycine-serine index did not relate as well to clinical outcome as did the glycine index. In contrast to control subjects, patients with NKH do not have raised serine levels after a glycine load (Cole and Meek 1985; Palmer and Oberholzer 1985; Steiman et al 1979). A serine load had a variable effect on the glycine level. For instance, in patient 5, a 300 mg/kg serine load administered in the morning raised the glycine level from 247 to 665 μmol/L after 3 h, but in patient 1, serine administered fasting in the morning did not raise the plasma glycine level (from 456 to 437 μmol/L). Similar variable responses of glycine levels to a serine load have been reported previously (Palmer and Oberholzer 1985; Steiman et al 1979; Trauner et al 1981). More standardized testing, preferably using stable isotope label studies, will be required to evaluate the contribution of serine to the glycine index in NKH patients.

Some parameters that make up the variability in the amount of benzoate required may be related to parameters that can potentially influence disease severity. Indeed, the glycine index, devised to describe this relation, correlated well with clinical severity as measured by neurodevelopmental outcome and by severity of seizure disorder, more so than the age of onset. In two surveys of NKH families, up to 20% of neonatally presenting patients made substantial developmental progress in learning to sit, walk and communicate (Hennermann et al 2001; Hoover-Fong et al 2004). Milder mutations and residual activity of the glycine cleavage enzyme were reported in two patients with a neonatal presentation with a better than expected outcome (Kure et al 2004). A more formal study relating mutations in

components of the glycine cleavage enzyme system to outcome or to the glycine index is still in progress. The main synthetic pathway of glycine is derived from serine. Common polymorphic mutations in the serine hydroxymethyltransferase genes, such as a 4 bp deletion delTCTT1721–1724 in the mitochondrial enzyme and the 1420C>T mutation in the cytosolic enzyme (Heil et al 2001), can influence glycine synthesis and should be investigated as possible factors modifying severity.

In conclusion, the glycine index is a stable, individually specific parameter in patients with NKH. It has clinical consequences for the dose of benzoate used and the role of diet. Through its correlation with neurodevelopmental outcome, the glycine index points to potential genetic factors that can contribute to the psychomotor retardation in NKH.

ACKNOWLEDGEMENTS

There were no conflicts of financial interest. The authors express their gratitude to the participating patients and their parents.

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