J Inherit Metab Dis (2008) 31:67–72
DOI 10.1007/s10545-007-0572-4

ORIGINAL ARTICLE

Classifying tetrahydrobiopterin responsiveness in the hyperphenylalaninaemias

U. Langenbeck

Received: 1 February 2007 / Submitted in revised form: 19 September 2007 / Accepted: 22 November 2007 /
Published online: 22 January 2008

Summary
Background A significant percentage of patients with hyperphenylalaninaemia (HPA) due to primary deficiency of the phenylalanine hydroxylase enzyme (PAH) respond to a dose of tetrahydrobiopterin (BH_4) with an increased rate of phenylalanine (Phe) disposal. The effect is exploited therapeutically, with some patients on BH_4 even tolerating a normal diet.
Aim Classification of the Phe blood level response to a BH_4 load by percentage reduction (PR) suffers from loss of information: only part of usually more extensive test data is used, and PR values for different times after load cannot be compared directly. Calculation of half-life ($t_{1/2}$) of blood Phe is proposed as an alternative. This classic measure unifies interpretation of tests of different duration (e.g. 8 or 15 h). $t_{1/2}$ subsumes first-order formation of tyrosine, of Phe metabolites, and renal Phe excretion; zero-order net protein synthesis can be neglected during short-time tests.
Method $t_{1/2}$ is easily and robustly obtained by fitting the *total set* of (3–4) data points to a log-linear regression.
Results The advantage of calculating $t_{1/2}$ is exemplified by the analysis of selected published data. The results clearly speak in favour of an 8 h test period because so-called 'slow' responders could also be detected within this time window and because tests of longer duration are less reliable kinetically. Sequential Phe and Phe/BH_4 loading tests appear advantageous because the 'natural' $t_{1/2}$ (without supplementation of BH_4) is not normally known beforehand.
Conclusion With $t_{1/2}$ as a reliable parameter of BH_4 responsiveness, therapeutic decisions would be more rational and genotype–phenotype analysis may also profit.

Abbreviations

BH_4	5,6,7,8-tetrahydro-l-biopterin
HPA	hyperphenylalaninaemia
k_e	first-order rate constant of elimination
MHP	mild hyperphenylalaninaemia
PAH	phenylalanine hydroxylase (EC 1.14.16.1)
Phe	phenylalanine
PKU	phenylketonuria
PR	percentage reduction of blood Phe during BH_4 load
RR	relative reduction of blood Phe during BH_4 load
SED	single exponential decay

Communicating editor: Johannes Zschocke

Competing interests: None declared

References to electronic databases: Phenylketonuria: OMIM +261600. Phenylalanine hydroxylase: EC 1.14.16.1.

U. Langenbeck (✉)
Institute of Human Genetics, University Hospital,
Theodor-Stern-Kai 7, D-60590 Frankfurt/Main, Germany
e-mail: u.langenbeck@em.uni-frankfurt.de

Introduction

Since 'a new molecular defect in phenylketonuria' (PKU) was recognized (Bartholomé 1974; Smith and Lloyd 1974), these disorders of biopterin metabolism

were screened for, in addition to other methods, with an oral or intravenous BH_4 load at elevated plasma concentrations of Phe (Curtius et al 1979; Danks et al 1978, 1979). In consideration of the paradigm of cofactor-responsive metabolic disorders without cofactor deficiency (Rosenberg 1976), it was expected from the outset (Curtius et al 1979) that such a test would also detect cofactor 'K_M-mutants' of the phenylalanine hydroxylase (PAH) enzyme itself. None, however, was reported during the next decade (Niederwieser et al 1985). In retrospect, this is not surprising because diagnosis of malignant hyperphenylalaninaemia (HPA) required the normalization of plasma Phe within 6 h after administration of 2 mg tetrahydrobiopterin (BH_4) per kg body weight (Danks et al 1979). It is only since 1999 that BH_4-responsive primary PAH deficiency has been described in the literature (Kure et al 1999). This first publication immediately triggered therapeutic research (Muntau et al 2002; Steinfeld et al 2002; Trefz et al 2001), and it could be demonstrated that most individuals with mild hyperphenylalaninaemia (MHP), a high proportion of patients with mild PKU and even some patients with classic PKU manifest an increased Phe tolerance while taking BH_4 (Bernegger and Blau 2002; Desviat et al 2004; Fiege and Blau 2007). Treatment with BH_4 even may substitute in some patients for diet therapy (Bélanger-Quintana et al 2005; Boneh et al 2006). The clinical studies resulted in the Orphan Drug Designation of (6*R*)-BH_4 (sapropterin dihydrochloride, Phenoptin) in the European Union in 2004 (EMEA 2007).

A responsible selection of patients eligible for BH_4 therapy (with no false-negatives and false-positives) will now require unified and methodologically sound procedures for evaluation of BH_4-responsiveness to identify all the patients who would benefit from this new therapeutic approach. In the present report, a kinetic approach with calculation of blood Phe half-life $t_{1/2}$ is proposed as such a procedure. This classic nonlinear kinetic parameter is determined routinely in clinical pharmacology (Ritschel 1982) and has also been applied in the field of inborn errors of metabolism (Schadewaldt et al 1991; Snyderman et al 1964). As exemplified by re-analysis of BH_4 test data from published records (Desviat et al 2004; Fiori et al 2005; Fiege et al 2005; Habich 2006; Lindner et al 2003; Muntau et al 2002), this method is practicable, robust and reliable. Also through this analysis, the advantages of a short (8 h) test duration and of a sequential Phe load without and with BH_4 are demonstrated.

Current methods of studying BH_4 responsiveness in hyperphenylalaninaemia

Methods of BH_4 loading

The published loading tests for identifying BH_4-responsive patients may be systematized according to (i) the administered BH_4 preparation (~70% vs 100% (6*R*)-BH_4), (ii) the patient's age at test (newborn vs older), (iii) the time of test surveillance (short-term (8, 12, 15 h) vs long-term (24, 48 h) vs very long-term (8 days)), (iv) single BH_4 or Phe plus BH_4 vs sequential Phe and Phe plus BH_4 dosage, (v) dosage scheme of BH_4 (10 or 20 mg BH_4/kg body weight once or twice or daily), and (vi) the dietetic conditions during the test (controlled addition of Phe after the beginning of the test vs continuation of individual normal or Phe-reduced diet); see Zurflüh et al (2006) and Fiege and Blau (2007).

Analysis of BH_4 loading test results

Most authors report the response to BH_4 as percentage reduction (PR) of plasma Phe at defined hours after administration of BH_4, i.e. 8, 12, 15, 24 or 48 h. Usually, however, more than just the two Phe blood levels for calculation of PR are obtained during the test, and they remain largely unused. This loss of information is augmented by the procedure itself, because the nonlinear process of Phe-disposal cannot be described meaningfully with the linear PR term. Accordingly, a direct comparison of PR values obtained at different points in time is not possible. A somewhat better parameter is the dimension-less slope *S*, of Bernegger and Blau (2002), as calculated from the slopes at 0–4 and 4–8 h post load. But this is still a linear approach and the time to reach a target concentration of Phe must be read from a curved plot of empirical data.

Interpretation of blood Phe response

In contrast to the disorders of BH_4 synthesis, response of blood Phe after administration of BH_4, as a rule, is only partial in PAH deficiency. This raises the

question of how to define responsiveness. 'Under ideal conditions' (Blau and Erlandsen 2004), the most reliable, controlled approach would be to load the patients twice, first with Phe alone and thereafter with Phe plus BH_4. However, such data have been published only rarely (Desviat et al 2004; Porta et al 2007); see below. Most studies consider responsiveness as proven if some arbitrary threshold of PR is exceeded, e.g. 30% at 8 h (Bernegger and Blau 2002, Fiori et al 2005) and 15 h (Muntau et al 2002), or 50% at 24 h (Fiege et al 2005). The subdivision of the response into 'fast', 'moderate', 'slow', and 'none' has resulted in more elaborated classification schemes which try to reproduce the various time courses of blood Phe (Fiege et al 2005; Zurflüh et al 2006). They pose the problem that either the response remains 'undefined' by definition in some combinations of test results (Zurflüh et al 2006) or that patients, within the same test, are 'responsive' as well as 'unresponsive' (Fiege et al 2005). By the scheme of Bernegger and Blau (2002), loading tests with $S > 3.75$ (see above) are considered significantly positive. This value corresponds to a $t_{1/2}$ of about 12 h (12.5 h are needed to lower blood Phe from 750 to 360 μmol/L).

Kinetic analysis of the BH_4 response

Disposal of Phe after complete equilibration in the body fluids (3–4 h after the load, see Blau and Erlandsen 2004) can be described in a first approximation as single exponential decay (SED) which subsumes first-order synthesis of tyrosine and secondary Phe metabolites, and urinary excretion, and ignores (net) zero-order protein synthesis (Langenbeck et al 2001). So the simplest and most illustrative parameter for characterizing the kinetics of Phe decline is the half-life $t_{1/2}$ of plasma Phe. With only few (3–4) data points as in BH_4 loading tests, it is obtained easily through log-linear regression of Phe on time, with $t_{1/2} = -(\log 2)/\text{slope}$ on using the decadic logarithm, or with $t_{1/2} = -(\ln 2)/\text{slope}$ on using the natural logarithm of blood Phe. This method of unweighted linearization, however, is prone to errors from outliers in the nonlinear process. Therefore, with more than 3–4 time points per series, analysis as SED with Marquardt algorithm (contained, e.g., in the kinetic software packages EnzFitter of Biosoft, Cambridge, UK, and Scientist of MicroMath, St. Louis, MO, USA) will yield an optimized estimate of k_e for the total set of test data, including possible outliers. It is assumed in all the following calculations that the activating effect of administered BH_4 on Phe disposal is an instant and maximal one.

With $t_{1/2}$ known, it is possible to calculate the time T it takes to reach a given blood concentration C_T of Phe at time t_2, starting at time t_1 from any initial concentration C_0:

$$\frac{\log C_0 - \log C_T}{t_2 - t_1} = -\text{slope} = \frac{\log 2}{t_{1/2}} \quad (1)$$

$$T = t_2 - t_1 = \frac{\log C_0 - \log C_T}{\log 2} \times t_{1/2} \quad (2)$$

Equation 2, however, is valid only for the dietetic conditions under which $t_{1/2}$ was determined. It is plotted in Fig. 1 for three different values of C_0, a set of four therapeutically relevant values of $t_{1/2}$, and a C_T of 360 μmol/L. This linear, theoretical plot is analogous to the nonlinear plot of Bernegger and Blau

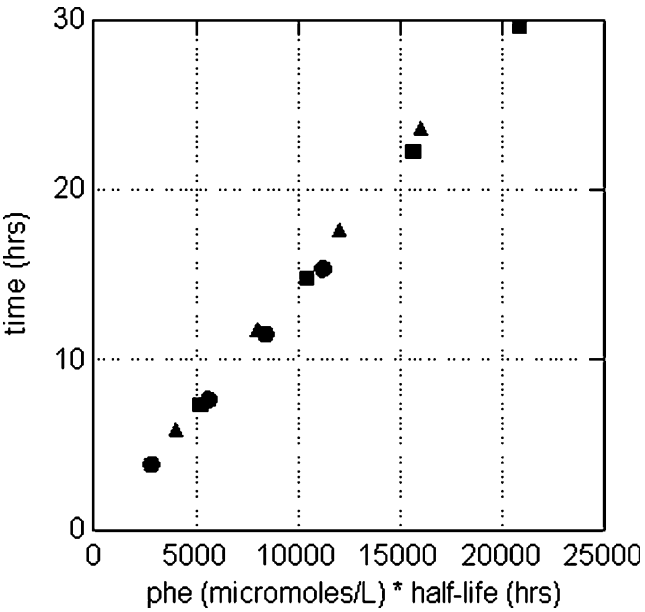

Fig. 1 The time T it takes to reach a Phe blood level C_T of 360 μmol/L given initial Phe blood levels C_0 of 700 μmol/L (circles), 1.000 μmol/L (triangles), and 1.300 μmol/L (squares), and half-lives $t_{1/2}$ of 4, 8, 12, and 16 h, respectively The points were calculated using equation 2. For each C_0, the points correlate with $r = 1$. Taking all points together ($r = 0.9987$), the error in predicting the time T as $T = (\text{initial Phe} \times \text{half-life})/690$ does not exceed 5%.

(2002) in their figure6, where the initial Phe/slope S ratios are plotted against the empirical time to reach a Phe level of 360 μmol/L.

The percentage reduction (PR) of blood Phe at time T as a function of $t_{1/2}$ is obtained as follows (RR = relative reduction):

$$C_T = C_0 e^{-k_e T} = C_0 e^{-(\ln 2)T/t_{1/2}} \quad (3)$$

$$C_0 - C_T = C_0 - C_0 e^{-(\ln 2)T/t_{1/2}} \quad (4)$$

$$\mathrm{RR} = (C_0 - C_T)/C_0 = 1 - e^{-(\ln 2)T/t_{1/2}} \quad (5)$$

Equation 5, by rearrangement and with PR = RR × 100, yields:

$$t_{1/2} = -T(\log 2)/\log(1 - \mathrm{RR}) \quad (6)$$

$$\mathrm{PR} = \left(1 - e^{-2.3026(\log 2)T/t_{1/2}}\right) \times 100 \quad (7)$$

This relation allows comparison of PR data for different test times, see Fig. 2 below.

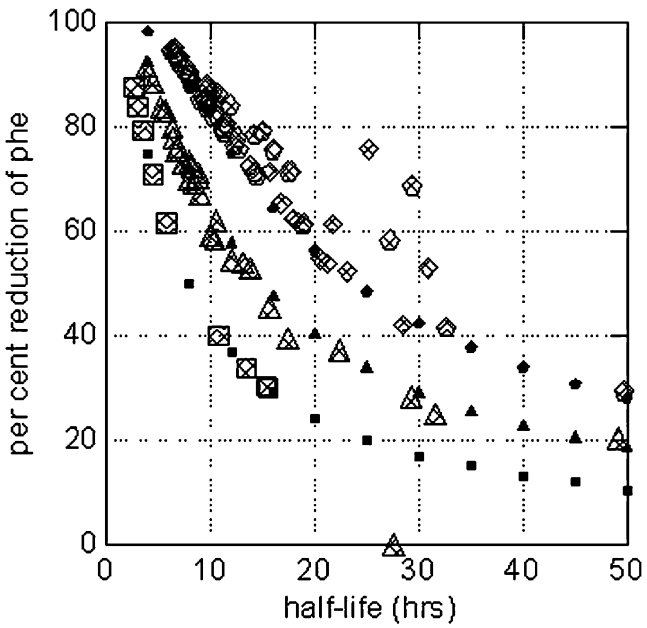

Fig. 2 Relation between half-life $t_{1/2}$ and percentage reduction (PR) of plasma Phe after 8 h (squares), 15 h (triangles), and 24 h (pentagons), respectively. Filled symbols are model data of first-order elimination with no uptake of additional Phe after beginning of the test (see text). Open symbol data result from re-analysis of the tests of Lindner et al (2003) (8 h), Muntau et al (2002) and Habich (2006) (15 h), Fiori et al (2005) (24 h, pentagons) and Fiege et al (2005) (24 h, diamonds). The data with $t_{1/2} > 50$ h and/or negative PR are excluded. The outliers between 25 and 31 h $t_{1/2}$ are artefacts because plasma Phe had increased again at 24 h.

Results

In order to demonstrate the utility of $t_{1/2}$ for classifying the BH_4 response, I re-calculated the test results of Lindner et al (2003), Muntau et al (2002) (see the thesis of Habich (2006) for the original data), Fiori et al (2005) and Fiege et al (2005) with log-linear regression and compared the results with the model of SED of blood Phe. It can be seen from Fig. 2 that only during short tests (8 and 15 h, respectively) does the kinetic behaviour of plasma Phe obey first-order kinetics. Within the half-time window of 0–50 h, the correlation between expected PR (as calculated with equation 7) and observed PR is $r = 0.9999$ at 8 h ($n = 9$, Lindner et al 2003), and 0.9955 at 15 h ($n = 30$, disregarding the outlier at $t_{1/2} = 28$ h, i.e. patient 28 of Muntau et al (2002) and Habich (2006)). In contrast, with a test duration of 24 h, only part of the data is still compatible with the model. Also, as an indication of incipient net protein synthesis, even the data close to the model almost always have higher PRs than predicted. If a PR of 30% at 24 h (corresponding to a $t_{1/2}$ of about 50 h) is taken as threshold for responsiveness (Fiege and Blau 2007), a test duration of 15 h would serve the purpose equally well because the corresponding PR of about 20% is safely within the margins of analytical accuracy.

It is of interest to relate the customary grades of responsiveness to the matching $t_{1/2}$ values. Muntau et al (2002) distinguish 'yes', 'moderately' and 'no'. This classification corresponds to $t_{1/2}$ values (median, range) of 8.0 (3.9–13.8; $n = 23$), 16.6 (10.4–22.4; $n = 4$) and 31.6 h (27.6–323.3; $n = 9$), respectively. Fiege et al (2005) distinguish 'rapid', 'moderate', 'slow' and 'non-resp.'. Using their 0–8 h test data only, these correspond to $t_{1/2}$ values (median, range) of 6.6 (1.6–14.0; $n = 10$), 10.6 (5.6–17.8; $n = 4$), 45.4 (29.6–73.2; $n = 4$) and 149.4 h (75.8–211.4; $n = 5$), respectively. The 'natural' $t_{1/2}$ values (without additional BH_4) are not known for these patients. For comparison, $t_{1/2}$ was determined by intravenous Phe loads as 13.6, 25.2 and 46.2 h for 'mild', 'atypical', and classic PKU, respectively (Rey et al 1979). Thus, the nonresponders of Muntau et al (2002) and the 'slow responders' of Fiege et al (2005) behave kinetically like the patient with classic PKU of Rey et al (1979), whereas some of the 'rapid responders' are borderline normal: Four determinations of normal $t_{1/2}$ by intravenous Phe load range from 0.67 to 1.47 h, see Langenbeck and Wendel (1997) for references.

The more extensive test data of Desviat et al (2004) with 4–5 time points per series were re-calculated by SED with Marquardt algorithm, as contained in the

Table 1 Half-life (h) of blood phenylalanine with and without BH_4. The data of Desviat et al (2004) are re-calculated with log-linear regression of table2 data from0–20 h and of table3 data from0–16 h. The relative standard error of k_e (SE/k_e) is given in parentheses. Patient no.19548 may have been in a catabolic state during the test

Clinical type	Patient no.	With BH_4	Without BH_4
MHP	12710	6.2 (0.037)	18.8 (0.119)
	18801	3.8 (0.260)	22.9 (0.482)
	18447	8.6 (0.147)	–
	18811	6.3 (0.431)	11.6 (0.146)
	18930	4.9 (0.144)	15.1 (0.138)
	19548	16.2 (0.219)	No decay
Mild PKU	11332	23.8 (1.20)	
	12528	9.9 (0.177)	
	12576	36.4 (0.504)	
	12895	24.3 (0.721)	
	18236	18.5 (0.094)	
	19068	6.8 (0.114)	
Moderate PKU	12324	31.6 (0.391)	
Classical PKU	10637	23.7 (0.145)	

ENZFITTER software (Leatherbarrow 1987). This yielded optimized estimates of k_e from which the $t_{1/2}$ data in Table 1 are obtained. The relative standard errors of k_e with the SED/Marquardt method and of the slope with log-linear regression (median and range, $n = 18$) are 0.162 (0.037–1.205) and 0.181 (0.054–2.287), respectively.

A comparison of the tests without and with BH_4 directly conveys the meanings of responsiveness and therapeutic value. However, also longer half-lives of Phe are significant therapeutically as demonstrated by patients no. 11332 and 12895 who tolerated a free diet with 10 and 15 mg BH_4/kg per day, respectively (Bélanger-Quintana et al 2005). This raises the interesting question of possible long-term effects of BH_4 on stability and/or synthesis of mutant PAH enzymes, as discussed below.

Discussion

In the present report, selected published BH_4 loading tests are re-evaluated under the model of first-order kinetics, with the explicit assumption that the disposal of Phe is increased instantly and with maximal effect. The molecular basis of such a response could be a chaperone effect of the cofactor on the available mutant enzyme molecules or its pharmacological effect on a K_M mutant of PAH. However, in addition to these mechanisms, possible effects on protein synthesis also are discussed in the literature (Blau and Erlandsen 2004; Erlandsen et al 2004). Whereas most data are compatible with a direct BH_4 effect, some are indicative of a long-term improvement of responsiveness (to be distinguished from the so-called 'slow response'). It could be explained by a growing number of stabilized PAH molecules. Examples are patients nos. 11332 and 12895 of Desviat et al (2004): they tolerated a free diet with 10 and 15 mg BH_4/kg per day, respectively (Bélanger-Quintana et al 2005) although their $t_{1/2}$ of ~24 h with BH_4 at time of test was closer to the range of 'atypical' PKU (Rey et al 1979) than to $t_{1/2}$ values of MHP patients without BH_4, see Table 1. Best documented is the long-term improvement of responsiveness with an extended three- to seven-day diagnostic study in patient no. 5 of Shintaku et al (2004).

Recently, the pragmatic approach of an eight-day diagnostic course has been reported (Trefz et al 2007). Comprehensive use of such extended diagnostic tests surely would minimize, with regard to BH_4 therapy, the number of false negatives. However, the prevalence of the true (and probably rare) late-responders and their molecular-genetic properties would remain unknown. Genotype–phenotype analysis will profit from a theory-driven diagnostic approach as proposed in the present paper. In addition, knowledge of individual $t_{1/2}$ values and age-specific protein synthesis could guide rational therapeutic decisions. In conclusion, a sequential Phe and Phe plus BH_4 dosage with accurate and precise determination of Phe at 0, 2, 4 and 8 h after administration of BH_4 might advance our knowledge of the pathogenesis and treatment of hyperphenylalaninaemia.

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