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DOI: <https://doi.org/10.1007/s10545-006-0425-6>

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Journal Article

Published Version

Originally published at:

Zurflüh, M R; Fiori, L; Fiege, B; Ozen, I; Demirkol, M; Gärtner, K H; Thöny, B; Giovannini, M; Blau, N (2006). Pharmacokinetics of orally administered tetrahydrobiopterin in patients with phenylalanine hydroxylase deficiency. *Journal of Inherited Metabolic Disease*, 29(6):725-731.

DOI: <https://doi.org/10.1007/s10545-006-0425-6>

Pharmacokinetics of orally administered tetrahydrobiopterin in patients with phenylalanine hydroxylase deficiency

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Received: 6 July 2006 / Submitted in revised form: 27 September 2006 / Accepted: 28 September 2006 / Published online: 7 November 2006
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Summary The oral loading test with tetrahydrobiopterin (BH₄) is used to discriminate between variants of hyperphenylalaninaemia and to detect BH₄-responsive patients. The outcome of the loading test depends on the genotype, dosage of BH₄, and BH₄ pharmacokinetics. A total of 71 patients with hyperphenylalaninaemia (mild to classic) were challenged with BH₄ (20 mg/kg) according to different protocols (1 × 20 mg or 2 × 20 mg) and blood BH₄ concentrations were measured in dried blood spots at different time points (T₀, T₂, T₄, T₈, T₁₂, T₂₄, T₃₂ and T_{48h}). Maximal BH₄ concentrations (median 22.69 nmol/g Hb) were measured 4 h after BH₄ administration in 63 out of 71 patients. Eight patients presented with maximal BH₄ concentrations ~44% higher at 8 h than at 4 h. After 24 h, BH₄ blood concentrations dropped to 11% of maximal values. This profile was similar using different protocols. The following pharmacokinetic parameters were calculated for BH₄ in blood: $t_{\max} = 4$ h, $AUC (T_{0-32}) = 370 \text{ nmol} \times \text{h/g Hb}$, and $t_{1/2}$

for absorption (1.1 h), distribution (2.5 h), and elimination (46.0 h) phases. Maximal BH₄ blood concentrations were not significantly lower in non-responders and there was no correlation between blood concentrations and responsiveness. Of mild PKU patients, 97% responded to BH₄ administration, while one was found to be a non-responder. Only 10/19 patients (53%) with Phe concentrations of 600–1200 µmol/L responded to BH₄ administration, and of the patients with the severe classical phenotype (blood Phe > 1200 µmol/L) only 4 out of 17 patient responded. An additional 36 patients with mild hyperphenylalaninaemia (HPA) who underwent the combined loading test with Phe+BH₄ were all responders. Slow responders and non-responders were found in all groups of HPA.

Abbreviations

AUC area under the curve
BH₄ tetrahydrobiopterin
HPA hyperphenylalaninaemia
PAH phenylalanine hydroxylase
PKU phenylketonuria
 $t_{1/2}$ half-life

Communicating editor: Alberto Burlina

Competing interests: None declared

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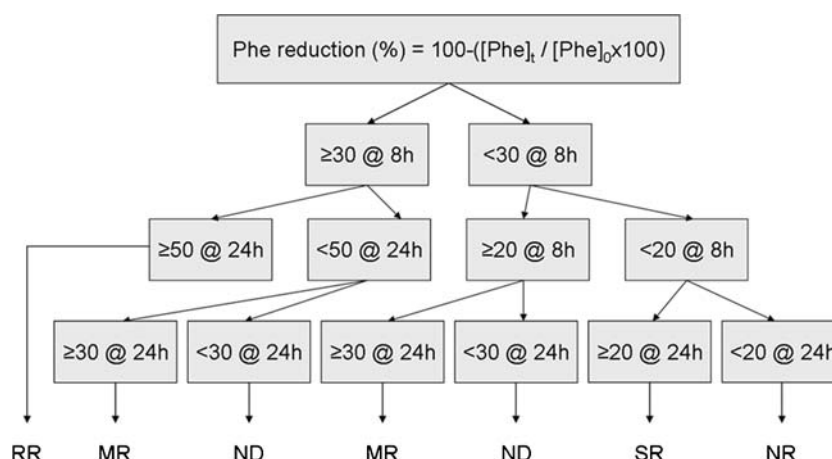
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Introduction

The loading test with tetrahydrobiopterin (BH₄) is an essential and integral part of the differential diagnosis for hyperphenylalaninaemia (HPA) (Blau et al 2001). This test discriminates between BH₄ responders and non-responders and is particularly important for detection of patients with BH₄-responsive phenylalanine hydroxylase (PAH; EC 1.14.16.1) deficiency (Blau and Erlandsen 2004). In BH₄ responders, blood phenylalanine (Phe) declines 4–24 h after BH₄ administration (20 mg/kg body weight), with almost complete

Fig. 1 Definition of the BH₄ responsiveness after oral administration of BH₄ (20 mg/kg). RR: rapid responder; MR: moderate responder; SR: slow responder; NR: non-responder; ND not defined



normalization after 4–8 h in patients with BH₄ deficiency. BH₄-responsive PAH patients were initially defined as showing a decrease of blood Phe concentrations of 30% after 8 h and 30–50% after 24 h (Bernegger and Blau 2002) and most of them belong to the group of mild HPA and mild and moderate phenylketonuria (PKU; OMIM 262600) (Desviat et al 2004; Fiori et al 2005; Kure et al 1999; Lässker et al 2002; Lindner et al 2003; Lücke et al 2003; Matalon et al 2004; Mitchell et al 2005; Muntau et al 2002; Perez-Duenas et al 2004; Spaapen et al 2001; Steinfeld et al 2003; Trefz and Blau 2003). Sensitivity of the test was further improved by multiple administrations of BH₄ and by longer observation time (Fiege et al 2005; Shintaku et al 2004). Efficiency and interpretation of the loading test depends on several factors such as amount of administered BH₄, severity of HPA, dietary management, and genotype. The pharmacokinetics of BH₄ was suggested as an additional factor affecting the loading test; however, this was never investigated in patients with PKU (Fiege et al 2004).

The aim of this study was to estimate basic kinetic parameters for BH₄ in blood after administration of BH₄ and following the combined Phe+BH₄ loading test, and to correlate the BH₄ concentrations in blood with the outcome of the test.

Materials and methods

Patients

A total of 71 HPA patients in whom BH₄ deficiency had been excluded were loaded with a single dose of BH₄ (20 mg/kg); 35 of them presented with basal blood Phe concentrations <600 μmol/L, 19 with Phe concentrations of 600–1200 μmol/L, and 17 with Phe concentrations >1200 μmol/L. In 11 patients with blood Phe concentrations of 278–1575 μmol/L the standard test was extended by the administration of another 20 mg/kg BH₄ after 24 h. An additional 36

patients with basal Phe concentrations of 384–739 μmol/L underwent a combined Phe (100 mg/kg) and BH₄ (20 mg/kg) loading test.

Mutation analysis was done in only a few patients and was not included in this study. The study was performed after a formal consensus of patients or their parents and in accordance with the Helsinki recommendations 1989.

Loading test

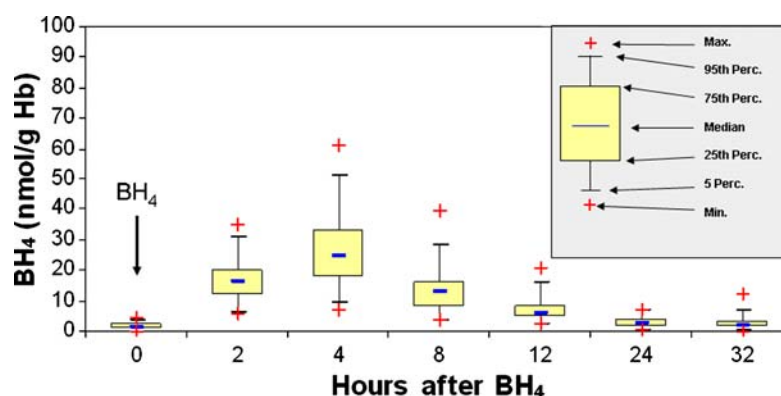
The single BH₄ test, the extended test, and the combined Phe+BH₄ loading test were performed as described previously using the 6R-BH₄ (Schircks Laboratories, Jona, Switzerland) (Blau and Erlandsen 2004; Fiege et al 2005). In patients loaded with a single dose of BH₄ (20 mg/kg), blood was collected at times T₀, T₄, T₈ and T_{24h}. In 45 of them additional blood collections were done at T₂, T₁₂ and T_{48h}. In patients loaded with 2 × 20 mg/kg BH₄, blood was collected at T₀, T₄, T₈, T₂₄, T₃₂ and T_{48h}, and in patients loaded with Phe+BH₄ the following blood collections were performed: T₋₃ (Phe administration), T₀ (BH₄ administration), T₄, T₈ and T_{24h}.

The following criteria were used to define BH₄-responsiveness over 24 h (Fiege et al 2005): 'rapid responder', reduction of blood Phe by ≥30% at T₈, and ≥50% at T₂₄; 'moderate responder', reduction of blood Phe by ≥20% at T₈, and ≥30% at T₂₄; 'slow responder', reduction of blood Phe by <20% at T₈, and ≥20% at T₂₄ (Fig. 1).

BH₄ and Phe in blood

BH₄ was measured in dried blood spots according to the method of Zurflüh and colleagues (2005) and calculated as the sum of total bipterin and pterin (nmol BH₄/g Hb). The following protocol was used: For each measurement, four blood circles (Ø6 mm) were cut out and pterins were extracted with 250 μL of 20 mmol/L HCl and placed in an ultrasonic bath for 30 s. Pterins were extracted by mixing the filter

Fig. 2 BH₄ concentrations in blood after oral administration of BH₄ (20 mg/kg) in 47 patients with HPA. — Median, □ 25th–75th percentiles; ⊥ 5th percentile; ⊤ 95th percentile; + min/max



spot solution for 10 min at room temperature. The extract was centrifuged at $1800 \times g$ for 5 min at room temperature. Clear supernatant (60 μ L) was used for analysis of haemoglobin on the haematology analyser Sysmex KX-21N (Sysmex Corporation, Japan). The remaining supernatant was ultrafiltered on Ultrafree (NMWL 10000; Millipore) at $5000 \times g$ for 15 min. Pterins were analysed in clear filtrate by HPLC and fluorescence detection without prior oxidation (Zurflüh et al 2005).

Phe was measured using standard ion-exchange chromatography of amino acids or tandem-mass spectrometry.

Statistical analyses

WinSTAT for Excel (v. 2003.1) was used for descriptive statistics and for regression analysis. Pharmacokinetic parameters were calculated using the PK Solutions software, v. 2.0 (Summit Research Services, Montrose, CO).

Results

BH₄ kinetics in blood

In 63 out of 71 patients with HPA loaded with 20 mg/kg BH₄, blood BH₄ concentrations reached highest values after 4 h (median = 22.65 nmol/g Hb; 5th–95th percentiles = 10.07–49.97 nmol/g Hb). Four hours later (T₈) BH₄ concentrations decreased by 42% (median = 13.61 nmol/g Hb; 5th–95th percentiles = 5.25–31.63 nmol/g Hb) and after 24 h BH₄ concentrations were only 11% of maximal values (median = 2.29 nmol/g Hb; 5th–95th percentiles = 0.67–5.35 nmol/g Hb). In 8 out of 71 patients, blood BH₄ concentrations were ~44% higher at 8 h than values at 4 h after administration and the highest single BH₄ value was 96.39 nmol/g Hb at T_{4h}.

In 45 of the above patients the blood BH₄ profile was investigated over 32 h after BH₄ administration (20 mg/kg) with

additional time points at T₂, T₁₂ and T_{32h} (Fig. 2). Two hours after BH₄ administration (median = 16.30 nmol/g Hb; 5th–95th percentiles = 6.84–28.44 nmol/g Hb) blood concentrations reached about 70% of the maximal concentrations found after 4 h. At 12 h (median = 6.15 nmol/g Hb; 5th–95th percentiles = 2.90–12.38 nmol/g Hb) and 32 h (median = 2.03 nmol/g Hb; 5th–95th percentiles = 0.51–5.47 nmol/g Hb) BH₄ blood concentrations were lower than those at 8 h (47% and 15%, respectively).

In 11 patients the protocol was extended to a second administration of BH₄ (20 mg/kg) after 24 h (Fig. 3). Blood was collected 8 (T₃₂) and 24 (T₄₈) hours after the second administration and compared with T₈ concentrations for BH₄ in blood (median = 15.37 nmol/g Hb; 5th–95th percentiles = 7.05–54.02 nmol/g Hb); T₃₂ values were 17% lower (median = 12.80 nmol/g Hb; 5th–95th percentiles = 7.41–38.54 nmol/g Hb). There was no significant difference in BH₄ concentrations at T₂₄ and T_{48h}.

Thirty-six patients with blood Phe concentrations of <336 μ mol/L were loaded first with Phe (100 mg/kg) and three hours later with BH₄ (20 mg/kg), and blood samples were collected before Phe administration (T₋₃), before BH₄ administration (i.e. 3 h after Phe loading; T₀), and 4, 8 and 24 h after BH₄ administration (T_{4–24}) (Fig. 4). BH₄ concentrations in blood increased in 26/36 patients after Phe administration by 111% (BH₄ at T₋₃, median = 0.30 nmol/g Hb; 5th–95th percentiles = 0.12–2.77 nmol/g Hb; BH₄ at T₀, median = 0.52 nmol/g Hb; 5th–95th percentiles = 0.16–4.43 nmol/g Hb). The following profile of BH₄ kinetics was similar as the one described for a single BH₄ administration, with maximal blood concentrations at T₄ (median = 22.01 nmol/g Hb; 5th–95th percentiles = 10.40–46.58 nmol/g Hb), 25% lower concentrations at T₈ (median = 16.46 nmol/g Hb; 5th–95th percentiles = 4.66–33.49 nmol/g Hb), and 90% lower concentrations at T₂₄ (median = 2.09 nmol/g Hb; 5th–95th percentiles = 0.55–6.84 nmol/g Hb). In 6 subjects maximal BH₄ concentrations were reached after 8 h.

Table 1 Summary of BH₄ loading tests (20 mg/kg) in 71 patients with PAH deficiency

	<i>n</i>	Initial blood Phe concentrations		
		<600 µmol/L (<i>n</i> = 35)	600–1200 µmol/L (<i>n</i> = 19)	>1200 µmol/L (<i>n</i> = 17)
Responder (total)	48	34	10	4
Rapid responder	34	26	6	2
Moderate responder	10	7	3	0
Slow responder	4	1	1	2
Non-responder	21	1	8	12
Not defined	2	0	1	1

Based on data obtained over 32 h after a single BH₄ administration (20 mg/kg) in 45 patients with HPA, basic pharmacokinetic parameters were calculated: $t_{\max}$ was 4 h, AUC (T_{0-32}) was 370 nmol × h/g Hb, and $t_{1/2}$ for absorption, distribution, and elimination phases was 1.1, 2.5, and 46.0 h, respectively.

Responsiveness to BH₄

Table 1 summarizes the results of the loading test with 20 mg/kg BH₄ in patients with HPA. Responsiveness was calculated according to criteria defined in Fig. 1. 34/35

patients (97%) with basal blood Phe concentrations <600 µmol/L responded to BH₄ administration, one patient was found to be a non-responder. Only 10/19 patients (53%) with basal blood Phe concentrations of 600–1200 µmol/L responded to BH₄ administration, and of the patients with the severe classical phenotype (blood Phe > 1200 µmol/L) only four patients responded (24%), and two of them were slow responders (Fig. 5). The lower the Phe at T_0 the higher the probability that a patient will respond to BH₄. Two patients could not be assigned to the above-mentioned criteria; one would have been positioned somewhere between moderate and slow responder and the other was at the level of a

Fig. 3 BH₄ concentrations in blood after oral administration of BH₄ (2 × 20 mg/kg) in 11 patients with HPA. Second dosage of BH₄ was administered 24 h after the first application. — Median, □ 25th–75th percentiles; ⊥ 5th percentile; T 95th percentile; + min/max

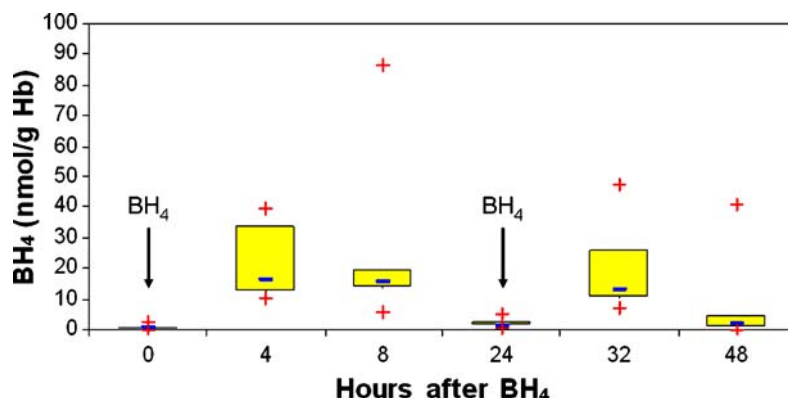


Fig. 4 BH₄ concentrations in blood after oral administration of Phe (100 mg/kg) and BH₄ (20 mg/kg) in 36 patients with mild HPA. BH₄ was administered 3 h after Phe application. — Median, □ 25th–75th percentiles; ⊥ 5th percentile; T 95th percentile; + min/max

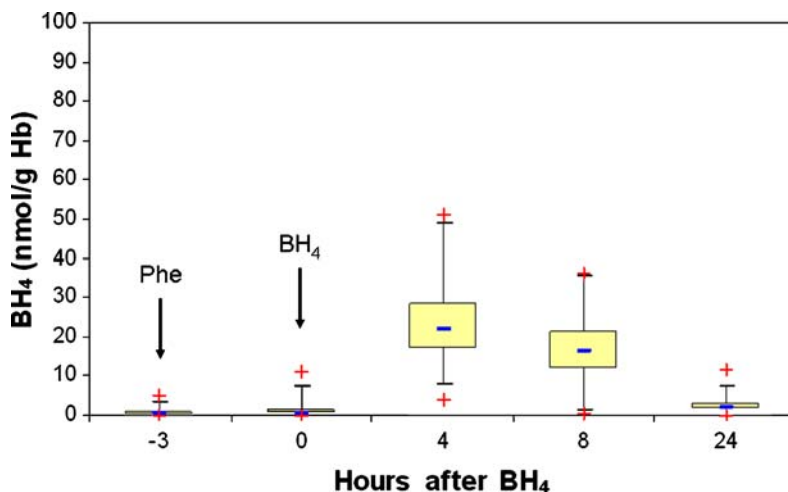


Fig. 5 Correlation between Phe decline in blood 24 h after administration of BH₄ (20 mg/kg) and basal Phe levels in 71 patients with HPA. ■ Rapid responder; ▲; moderate responder; * slow responder; · non-responder

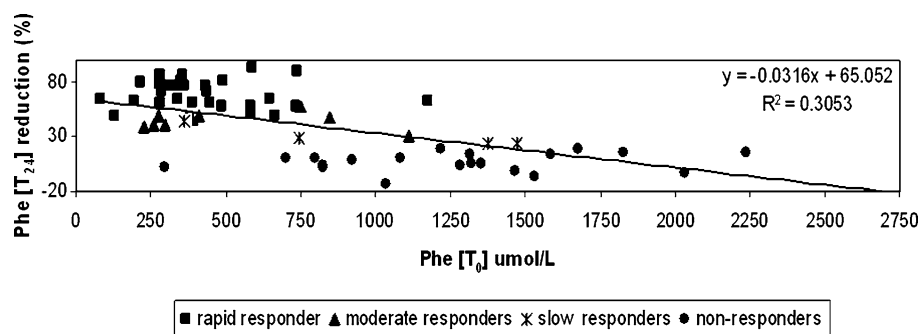
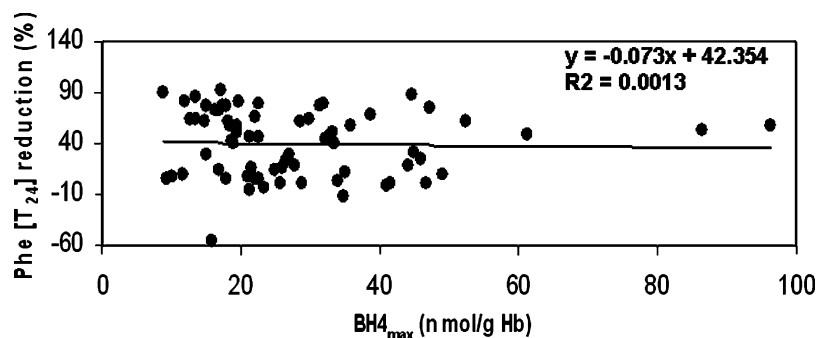


Fig. 6 Correlation between Phe decline in blood 24 h after administration of BH₄ (20 mg/kg) and maximal BH₄ levels in 71 patients with HPA



rapid responder after 8 h but dropped at 24 h below the level of a slow responder.

All 36 patients who underwent the combined loading test with Phe+BH₄ were classified as responders (data not shown).

In order to demonstrate whether absorption of BH₄ may affect the outcome of the loading test, maximal blood BH₄ concentrations were compared with responsiveness to BH₄, but no correlation was found (Fig. 6). Highest maximal blood BH₄ concentrations were found in two rapid responders.

Discussion

Extensive pharmacokinetic studies of BH₄ have been performed in animal models (Hayashi et al 1992), but only a few parameters are known from studies in humans (Fiege and Blau 2006). Some pharmacokinetic parameters are known from oral administration of BH₄ tablets to healthy adult human volunteers and might provide details on pharmacological response to BH₄ therapy (Fiege et al 2004). Plasma concentrations of BH₄ and total bipterin were assessed after oral administration of 6R-BH₄ at different doses to different healthy subjects and preliminary pharmacokinetic parameters have been determined (Fiege et al 2004). The plasma profile of total bipterin after oral administration exhibited first-order kinetics, showing a fast absorption phase (T₀–T₄), a rapid decline (T₄–T₁₀) corresponding to the absorption and distribution phase, followed by a slower decline in the final elimination phase (T₁₀–T_{33h}). Total bipterin concentrations in plasma have been studied after administration of different

doses (10 and 20 mg/kg) to one healthy adult subject (Fiege et al 2004). Maximal plasma concentrations in this subject were reached 4 h after the 10 mg/kg dose and 3 h after the 20 mg/kg dose, at concentrations of 258.7 and 441.7 nmol/L, respectively. The AUC_{0–10} after administration of 20 mg/kg was 1.6 times higher than the AUC after the 10 mg/kg dosage (3046 vs 1958 nmol h/L). Based on these data, the elimination kinetics seem to be only slightly faster at higher plasma concentrations (*t*_{max} = 4.2 h vs 5.1 h) (Fiege et al 2004).

Very little is known about BH₄ pharmacokinetics in patients with HPA. Shintaku and colleagues (2005) reported plasma bipterin concentrations in two patients with HPA who underwent a single BH₄ loading test (10 mg/kg) at different ages. In both patients plasma bipterin concentrations were ~100% higher at an early age (<1 month) compared with concentrations measured at the age of 2 months. Also, bipterin concentrations peaked at 4 h at the age of 1 month, compared with maximal concentrations at 2 h at the age of 2 months. The authors suggested that BH₄ responsiveness in the same individual or the same genotype may correlate with bipterin concentration, but in different genotypes this might not be the case (Shintaku et al 2005).

We were not able to see any statistical difference between different age groups in our patients (data not shown). Similarly to what was described for healthy controls (Fiege et al 2004), blood BH₄ peaked at 4 h in 90% of patients with HPA. This profile was consistent regardless of whether patients were loaded with one or two BH₄ doses or after Phe administration (Figs. 2–4). Two hours after BH₄ administration, blood concentrations were about 70% of the maximal

BH₄ concentrations, indicating a very fast absorption phase ($t_{1/2} = 1.1$ h). In our patients, oral administration of BH₄ resulted in a fast distribution phase ($t_{1/2} = 2.5$ h), followed by a slow elimination phase ($t_{1/2} = 46.0$ h). Thus, although a single BH₄ administration may be sufficient for the interpretation of the loading test, additional dosages can potentiate the effect and increase the sensitivity (Fiege et al 2005; Shintaku et al 2004).

Data from the combined Phe+BH₄ loading test show that administration of Phe (100 mg/kg) almost doubled blood BH₄ concentrations after 3 h in 72% of patients (Fig. 4). This is consistent with previous findings that bipterin concentrations in urine or plasma correlate with blood phenylalanine concentrations (Dhondt and Farriaux, 1982; Ponzone et al 1993) but have no consequences on the outcome of the loading test. Phenylalanine administration does not influence BH₄ concentrations upon oral BH₄ administration. As expected, all patients in this group were classified as responders and one should question how useful this test is. Factors such as spontaneous Phe elimination (Desviat et al 2004) or daily fluctuations (Leuzzi et al 2006) may influence the interpretation, and from our experience the combined loading test is not recommended. It can be only used in patients who are already on a strict low-phenylalanine diet with normalized blood phenylalanine concentrations.

One of the main goals of this study was to evaluate the effect of blood BH₄ concentrations on the outcome of the loading tests. Recently we described a single case with HPA (BH₄-responsive genotype) showing intra-individual variations in BH₄ absorption on two occasions, which resulted in different BH₄ blood concentrations and influenced the responsiveness (Zurflüh et al 2005). We were not able to repeat the loading test in non-responders in this study, but maximal BH₄ blood concentrations were not significantly lower in this group of patients. Indeed, maximal BH₄ blood concentrations were only 3% lower in non-responders than in all responders, and slow responders had 7% and 42% higher concentrations than moderate or rapid responders, respectively (data not shown).

With regard to the responsiveness to BH₄, our data confirm previous observations that rapid responders belong mainly to the groups of mild HPA and mild PKU, and that patients with classical PKU show either only a slow response or none at all (Fig. 5). Nevertheless, slow responders and non-responders were found in all groups of HPA.

Acknowledgements This work was supported by the Swiss National Science Foundation grant no. 3100000–107500/1.

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