

Extended tetrahydrobiopterin loading test in the diagnosis of cofactor-responsive phenylketonuria: A pilot study

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Abstract

Patients with tetrahydrobiopterin (BH₄)-responsive phenylalanine hydroxylase (PAH) deficiency may benefit from BH₄ therapy instead or in addition to the low-phenylalanine diet. Different loading test protocols are currently used to detect these patients. As a consequence, data on the rate of BH₄-responsiveness within patients with mild phenylketonuria (PKU) and/or more severe phenotypes show high variation and a more sensitive and standardised BH₄ loading test protocol needs to be defined. We modified the current standard BH₄ loading test (20 mg/kg) to a second administration of 20 mg/kg after 24 h and extended blood sampling to 48 h in 24 patients with PAH deficiency. Using this extended loading test (2 × 20 mg BH₄/kg), the rate of BH₄-responsiveness was calculated at 8, 24, and 48 h after BH₄ administration. We defined three groups of patients: “rapid responders” in 10/24 patients (4 mild HPA, 2 mild PKU, 2 moderate PKU, and 2 classic PKU), “moderate responders” in 4/24 patients (4 classic PKU), and “slow responder” in 4/24 patients (4 mild PKU). Six out of 24 patients (1 mild HPA, 1 moderate PKU, and 4 classic PKU) were found to be “non-responder.” Individual phenylalanine profiles show variations in responsiveness at different time points and sampling over 48 h was more informative than over 24 h in patients with mild and moderate PKU compared to mild HPA. Analysis of BH₄ loading tests in 209 patients with the standard BH₄ loading test protocol confirms only minor importance of the 24 h response: the rate of responsiveness to BH₄ after 24 h was shown to be equal to or even lower than after 8 h among most phenotypes. However, extension of the BH₄ loading test to 48 h and repeated BH₄ administration seems to be useful to detect BH₄-responsiveness in more severe phenotypes and allows detecting “slow responders” who may benefit from BH₄ therapy.

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Introduction

The rate of tetrahydrobiopterin (BH₄)-responsiveness was shown to be high among patients with phenylalanine hydroxylase (PAH) deficiency [1]. Particularly patients with mild forms of PAH deficiency respond to BH₄ administration by lowering their blood phenylalanine (Phe) levels. Thus, BH₄ may have an important role in pharmacological

therapy of hyperphenylalaninemia (HPA). Indeed, a number of PAH-deficient patients are reported to undergo BH₄ therapy at different doses and some of these patients relaxed or even discontinued dietary treatment [2]. Thus, a reliable BH₄ loading protocol, which ensures identification of all BH₄-responsive patients, is currently discussed. The currently used standard BH₄ loading test is defined by the administration of 20 mg BH₄/kg body weight and blood sampling at time points 0, 4, 8, and 24 h. Repeated administration of BH₄ and extension of blood sampling to 1 week were shown to influence responsiveness [3]. Furthermore, variables such as BH₄ pharmacokinetics, patients' age, and

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Phe intake during the test were shown to interfere with responsiveness to BH₄ [4] and responsiveness cannot be always predicted by the genotype [5]. The molecular mechanism for BH₄-responsiveness seems to be multifactorial [6]. Thus, the loading test needs to be sensitive and accurate enough to detect all potential responders. In this pilot study, we investigated responsiveness to BH₄ at 8, 24, 32, and 48 h after extended BH₄ administration (2 × 20 mg/kg at times 0 and 24 h), to optimise the BH₄ loading test protocol. Furthermore, we retrospectively evaluated the efficiency of the standard 24 h test.

Patients and methods

Twenty-four patients affected by PAH deficiency, referred to three different centres for diagnosis and treatment of HPA in Italy (9/24) and Switzerland (15/24) were investigated for responsiveness to BH₄ administration after an extended loading test protocol: 6R-BH₄ (Schircks Laboratories, Jona, Switzerland) was administered in two doses of 20 mg/kg at 0 and 24 h. Plasma amino acids and pterins were analysed at 0, 4, 8, 12, 24, 32, and 48 h after the first BH₄ administration. Responsiveness to oral administration of BH₄ was defined as: “rapid responder”: decline of plasma Phe levels at 8, 24, and 48 h of ≥30, ≥50, and ≥50%, respectively; “moderate responder”: decline of plasma Phe levels at 8, 24, and 48 h of ≥20, ≥30, and ≥50%, respectively; and “slow responder”: decline of plasma Phe levels at 8, 24, and 48 h of <20, ≥20, and ≥30%, respectively. To analyse responsiveness among different phenotypes patients were stratified on the basis of their Phe tolerance (daily Phe intake keeping blood Phe below 360–400 μmol/L) in four classes: mild HPA (MHPA; >600 mg Phe/day), mild PKU (miPKU; 400–600 mg Phe/day), moderate PKU (moPKU; 350–400 mg Phe/day), and classic PKU (cPKU; <350 mg Phe/day) [7].

The extended BH₄ loading test was performed in adolescent patients with poor dietary control and in newborns with HPA (Table 1). BH₄ deficiency was excluded by analysis of neopterin and biopterin in urine and measurement of dihydropteridine reductase activity in dried blood spots. Blood Phe values were determined by conventional amino acid analysis. Genotype analysis was performed in 20 out of 24 patients [7a,7b].

Standard 24 h loading tests were performed between 2002 and 2005 in 209 patients with different severity of HPA referred from different medical centres in different countries.

Results and discussion

In 24 patients loaded with 2 × 20 mg/kg BH₄ the rate of BH₄-responsiveness was different depending on the criteria used to define BH₄-responsiveness. Using current definitions proposed by the European Metabolic Group [8], BH₄-responsiveness was not significantly different at different times of sampling: 54% (13/24) at 8 h, 52% at 24 h (12/23),

and 50% at 48 h (12/24). However, analysis of individual Phe profiles revealed variations depending on time points of investigation. According to the definition of responsiveness (see Patients and methods), patient 8 was a non-responder at 8 h and a responder at 24 and 48 h. In contrast, patient 17 was a responder at 8 h, a non-responder at 24 h, and a responder at 48 h. Two further patients, 10 and 24, were not responsive at 48 h after BH₄ administration, even if they had been previously responsive at time 8 and 24 h. These patients were classified as “moderate responders.” Patients 6, 13, 15, and 16 showed no response at 8 h and a delayed response at 48 h; these patients were defined “slow responders.” All other patients who were responders at 8 h were also responders at 24 and 48 h (Table 2).

Among seven patients showing basal plasma Phe levels <400 μmol/L (Table 2) four patients were responsive 8 h after BH₄ administration (“rapid responder”) and responsiveness was not significantly different at 24 and 48 h. Basal phenylalanine levels correspond to the biochemical phenotype as patients no. 1, 2, 3, 4, and 5 of this group underwent the BH₄ loading test at diagnosis of HPA and before dietary intervention. Three patients with basal plasma Phe levels <400 μmol/L were “non-responders” (no. 4 and 7) or “slow responders” (no. 6) to oral BH₄ supplementation. Mutations in the PAH locus of one of these non-responsive patients correspond to a moderate phenotype (no. 7), other genotypes (patients no. 1, 2, 3, and 5) to mild phenotype.

Four out of five patients with basal Phe levels between 400 and 800 μmol/L were responsive 8 h after BH₄ administration and all five were responsive at 24 and 48 h (Table 2). Patients 8 and 9 reached minimal plasma Phe levels at 32 h (182 and 68 μmol/L, respectively) and patient 11 showed a minimal plasma Phe level of 15 μmol/L 8 h after BH₄ administration. Patient 10 showed responsiveness at 8 and 24 h, but not at 48 h. He was classified as a “moderate responder.” Mutation analysis among these patients evidenced mild and moderate BH₄-responsive PKU genotypes (Table 1, patients no. 9 and no. 12).

Among patients with plasma Phe values between 800 and 1000 μmol/L none fulfilled the criteria for “rapid” responsiveness, neither at 8 h (Phe reduction 7.6–17.1%), nor at 24 h (Phe reduction 2.6–26.4%), or at 48 h (Phe reduction 14.9–45.9%) (Table 2). Patient no. 13 had a 61% Phe decline 8 h after the second BH₄ administration (time 32 h), which subsequently decreased to 40.9% at 48 h. Three patients (no. 13, 15, and 16) were classified as “slow responders.”

A consistent rate of responsiveness to oral BH₄ administration was found among patients exhibiting more severe phenotypes (Phe >1000 μmol/L) (Table 2). In the group of patients with basal Phe values ranging between 1000 and 1200 μmol/L, two patients were responsive at 8, 24, and 48 h (patients no. 18 and 20). Patient no. 18 reached therapeutic levels (228 μmol/L) at 48 h, starting from basal Phe levels of 1113 μmol/L, and patient no. 17, responsive at time 8 h, showed only minimal Phe reduction from 8 to 24 h (from 32.8 to 42.1%). He was a responder at 48 h (Phe reduction >50%) and classified as “moderate responder.” Patient 19

Table 1

Initial phenylalanine (Phe) levels in blood, Phe tolerance, phenotypes, genotypes, and predicted and observed BH₄-responsiveness of patients loaded with 2 × 20 mg/kg BH₄

Pat. no.	Phe ^a NBS (μmol/L)	Phe ^b BH ₄ -test (μmol/L)	Phe tolerance (mg/day)	Phenotype ^c	Allele 1 (residual activity) ^d	Allele 2 (residual activity) ^d	Responder type	BH ₄ -responsiveness ^e						
								8 h(>20%)	8 h(>30%)	24 h(>20%)	24 h(>30%)	24 h(>50%)	48 h(>30%)	48 h(>50%)
1	275	385	400	miPKU	A403V (32%)	R408W (3%)	Rapid	+	+	n.d.	n.d.	n.d.	+	+
2	200	378	?	MHPA	V245A (51%)	V388M (43%)	Rapid	+	+	+	+	+	+	+
3	276	278	>600	MHPA	IVS10nt + 1G > A (?)	A300S (2%)	Rapid	+	+	+	+	+	+	+
4	222	296	>600	MHPA	n.d.	n.d.	Non- resp.	–	–	–	–	–	–	–
5	198	362	>600	MHPA	E390G (70%)	A403V (32%)	Rapid	+	+	+	+	+	+	+
6	954	370	500	miPKU	D222fs (<1%)	IVS10nt-3C > T ^g	Slow	–	–	+	–	–	+	–
7	1300	365	350	moPKU	L48S (39%)	R408W (3%)	Non- resp.	–	–	–	–	–	–	–
8	500	548	240	cPKU	IVS4nt-5C > G ^f	IVS10nt-11G > A	Moderate	+	–	+	+	+	+	+
9	492	439	411	miPKU	G272X (<1%)	P211T (72%)	Rapid	+	+	+	+	+	+	+
10	375	408	300	cPKU	E390G (70%)	A395P (15%)	Moderate	+	+	+	+	+	+	–
11	432	486	266	cPKU	n.d.	n.d.	Rapid	+	+	+	+	+	+	+
12	504	448	381	moPKU	R261Q (30%)	M276V (?)	Rapid	+	+	+	+	+	+	+
13	—	841	571	miPKU	n.d.	n.d.	Slow	–	–	+	–	–	+	–
14	114	832	206	cPKU	P281L (<1%)	P281L (<1%)	Non- resp.	–	–	–	–	–	–	–
15	1237	900	400	miPKU	F39del (20%)	F39del (20%)	Slow	–	–	+	–	–	+	–
16	1340	984	400	miPKU	F39del (20%)	F39del (20%)	Slow	–	–	+	–	–	+	–
17	650	1158	240	cPKU	R261Q (30%)	R261Q (30%)	Moderate	+	+	+	+	–	+	+
18	990	1076	600	MHPA	L48S (39%)	L48S (39%)	Rapid	+	+	+	+	+	+	+
19	1500	1125	300	cPKU	R158Q (10%)	Y277D (<1)	Non- resp.	–	–	–	–	–	–	–
20	800	1113	250	cPKU	R261Q (30%)	R261Q (30%)	Rapid	+	+	+	+	+	+	+
21	800	1545	270	cPKU	R261Q (30%)	Y356X (<1%)	Non- resp.	–	–	–	–	–	–	–
22	800	1469	200	cPKU	n.d.	n.d.	Non- resp.	–	–	–	–	–	–	–
23	200	1733	350	moPKU	IVS4nt-5C > G ^f	Y356X (<1%)	Rapid	+	+	+	+	+	+	+
24	1221	1595	114	cPKU	IVS10nt+1G > A (?)	R261X (<1%)	Moderate	+	+	+	+	+	+	–

n.d., not determined; MHPA, mild HPA; miPKU, mild PKU; moPKU, moderate PKU; cPKU, classical PKU.

^a NBS, newborn screening.^b Before the loading test.^c Based on Phe tolerance [7].^d Residual activity (%) of recombinantly expressed proteins (measured or predicted).^e Based on the loading test.^f Responsive mutation according to Muntau et al. [5].^g Desviat et al. [10].

Table 2
Plasma phenylalanine (Phe) levels and percentage of plasma Phe reduction after oral administration of BH₄ (2 × 20 mg/kg at 0 and 24 h) in PAH-deficient patients

Patient number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Time 0 h	358	378	278	296	362	370	365	548	439	408	486	448	841	832	900	984	1158	1076	1125	1113	1545	1469	1733	1595
Time 4 h	134	215	200	297	53	356	353	493	195	248	150	243	631	885	783	893	1034	920	1130	1040	—	1532	1308	857
Time 8 h	42	108	158	306	31	343	340	401	70	152	15	238	713	895	746	893	778	725	1084	721	1505	1522	1008	736
Time 12 h	50	—	—	—	—	—	—	347	—	115	—	—	—	—	—	—	576	—	—	—	1450	—	—	—
Time 24 h	—	108	107	290	88	282	333	248	125	141	204	173	629	810	662	770	671	513	1014	514	1498	1583	650	750
Time 32 h	121	—	121	272	64	257	334	182	68	103	94	176	326	721	607	773	—	370	984	308	1435	—	230	—
Time 48 h	174	86	111	259	86	203	359	212	126	250	197	167	497	708	487	643	487	227	954	228	1239	1398	311	848
Phe reduction (%)	88,3	71,4	43,2	-3,4	91,4	7,3	6,8	26,8	84,1	62,7	96,9	46,9	15,2	-7,6	17,1	9,2	32,8	32,6	3,6	35,2	2,6	-3,6	41,8	53,9
Time 8 h	86,0	—	—	—	—	—	—	36,7	—	71,8	—	—	—	—	—	—	50,3	—	—	—	6,1	—	—	—
Time 24 h	—	71,4	61,5	2,0	75,7	23,8	8,8	54,7	71,5	65,4	58,0	61,4	25,2	2,6	26,4	21,7	42,1	52,3	9,9	53,8	3,0	-7,8	62,5	53,0
Time 32 h	66,2	—	56,5	8,1	82,3	30,5	8,5	66,8	84,5	74,8	80,7	60,7	61,2	13,3	32,6	21,4	—	65,6	12,5	72,3	7,1	—	86,7	—
Time 48 h	51,4	77,2	60,1	12,5	76,2	45,1	1,6	61,3	71,3	38,7	59,5	62,7	40,9	14,9	45,9	34,7	57,9	78,9	15,2	79,5	19,8	4,8	82,1	46,8

did not respond to oral BH₄ supplementation (Phe reduction 9.9–15.2%).

Two out of four patients (no. 23 and 24) with Phe levels >1200 μmol/l showed a significant decline in blood Phe levels 8 and 24 h after BH₄ administration (Table 2). At 48 h only patient 23 was a “rapid responder” according to our criteria. This patient reached therapeutic values (Phe <360 μmol/l) 8 h after the second BH₄ administration (32 h). Patient 24 showed a 53% Phe reduction at 24 h and a 46.8% Phe reduction at 48 h and was classified as a “moderate responder.” This is an adolescent patient with insufficient compliance to diet therapy. Mutation analysis of the *PAH* locus in this patient evidenced a classic PKU genotype (IVS10nt + 1G > A/R261X). Obviously, the IVS10nt + 1G > A mutations seems to be responsible for BH₄ responsiveness in patients 3 and 24.

Mutations on the *PAH* locus were identified in 20 HPA patients (Table 1). Genotypes were classified according to genotype–phenotype correlation [9]. In four BH₄-responsive patients (no. 2, 5, 18, and 20) genotypes corresponded to MHPA phenotypes. In one of these patients (no. 18) a homozygous genotype for the L48S mutation was identified while functional hemizygoty for the same mutation (L48S/R408W) was not associated with BH₄-responsiveness (patient no. 7). Another homozygous mutation (R261Q/R261Q) was associated with a moderate PKU phenotype (patient no. 17) who responded at time 8, 24, and 48 h. The same mutation was found in another patient with classic PKU who was responsive only at 8 and 48 h. Classical PKU genotypes (patients no. 14, 19, 21, and 22) were not associated to BH₄-responsiveness, even though patient no. 24 (IVS10nt + 1G > A/R261X) showed initial responsiveness at 8 and 24 h, but not at 48 h.

Results on BH₄-responsiveness in 209 PAH-deficient patients who underwent the 24 h standard BH₄ loading test (20 mg/kg) between 2002 and 2005 confirm previous reports of a high rate in the group of mild HPA (Phe 200–600 μmol/L) and mild PKU (Phe 600–1200 μmol/L) (Fig. 1). Surprisingly, the total rate of BH₄-responsiveness for each group was higher 8 h than 24 h after BH₄ administration. Only among three phenotype groups (Phe 600–700, 700–800, and 1600–1700 μmol/L) was responsiveness the same at 8 and 24 h. A small number of additional patients were detected by extending the loading test up to 24 h after BH₄ administration: in 2/8 patients with basal Phe levels of 1300–1400 μmol/L, the 24 h Phe value had not previously been responsive; among patients with basal Phe levels of 1700–1800 μmol/L one additional patient was responsive at 24 h.

In summary, responsiveness to the extended loading test at 8, 24, and 48 h after BH₄ administration is consistent in 18 out of 24 investigated HPA patients. The 8 h response seems to be reliably sensitive in revealing BH₄-responsive patients, whereas extension of blood sampling to 24 h after a single BH₄ administration yields no additional information. Multiple administration and extension of Phe determination after the second BH₄ administration seems to be more relevant than the 24 h response, and confirms and completes data on total rate of responsiveness as already

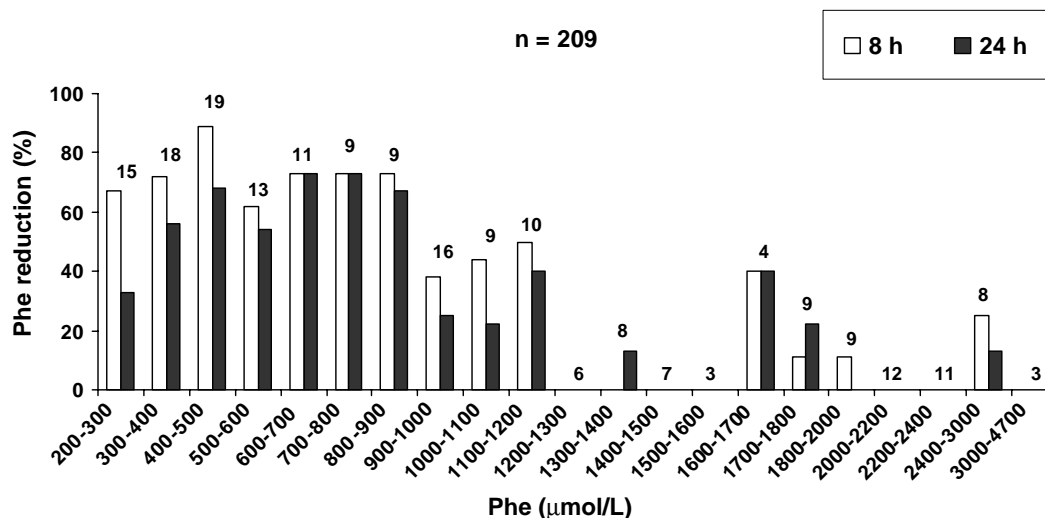


Fig. 1. Percentages of plasma Phe reduction 8 and 24 h after BH_4 administration (20 mg/kg) in patients with basal plasma Phe levels 200–4700 $\mu\text{mol/L}$. On the top of bars are the number of patients for individual HPA groups.

reported by Shintaku et al. [3]. Interestingly, some severe phenotypes were responsive 8, 24, and 48 h after BH_4 administration (patients no. 17, 18, 20, 23, and 24), which is in agreement with previous reports in literature [10–12]. Two patients of this group definitively reached therapeutic values (<360–400 $\mu\text{mol/L}$) after BH_4 administration (patients no. 18 and 20). In two other patients responsiveness 8 and 24 h after BH_4 administration was lower at 48 h (patients no. 10 and 24). These patients might be “moderate responders” and the 48 h response seems to be more reliable than previous values. Sampling at 48 h allowed identifying “slow” (or “moderate”) responders, who would have been classified as non-responders according to Phe levels at 8 and 24 h. Clinical follow-up under BH_4 therapy demonstrated that these patients indeed benefit from a sustained therapeutic effect of BH_4 , as they can increase their Phe tolerance of 50–100%, thus allowing a relaxed diet. BH_4 therapy may be important in these patients especially during adolescence, when compliance is less good, and during pregnancy to reach better controlled Phe levels.

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