

Plasma tetrahydrobiopterin and its pharmacokinetic following oral administration

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Abstract

Tetrahydrobiopterin (BH₄) is widely used as a therapeutic agent in patients with BH₄ deficiencies and mild forms of phenylketonuria (PKU) and there is an increasing need for the measurement of its plasma concentrations in patients with cardiovascular disorders. We measured BH₄ and total biopterin in dithioerythritol (DTE) pretreated plasma from four adults after oral administration of BH₄ (2, 10, and 20 mg/kg body weight) using the differential iodine oxidation method. About 80% (range 64.8–92.2%) of total biopterin was found as BH₄ when analyzed immediately after blood sampling. Compared with ascorbic acid as an antioxidant, DTE was more protective against oxidation of BH₄, particularly in samples stored over a period of 8 months. Without antioxidant (DTE or ascorbic acid) almost no BH₄ was detected. Furthermore, BH₄ and total biopterin were measured at different time intervals (up to 33 h after oral administration) and pharmacokinetic parameters T_{max} (1–4 h), C_{max} (258.7–259.0 nmol/L biopterin at a dosage of 10 mg/kg), and area under the curve (AUC = 1708–1958 nmol* h/L up to $T = 10$ h) were estimated. The elimination half-life time was calculated to be 3.3–5.1 h. Doubling the BH₄ dosage to 20 mg/kg resulted in 60% higher AUC while sublingual BH₄ application (2 mg/kg) resulted in 58–76% higher BH₄ plasma concentrations when compared with oral administration. These preliminary data suggest that in patients with BH₄ cofactor defects and BH₄-responsive phenylalanine hydroxylase deficiency, BH₄ should be given in at least two to three daily doses and that sublingual administration may lower the required BH₄ dosage and subsequently the cost of treatment. Due to inter individual differences in pharmacokinetic properties, in some patients with hyperphenylalaninemia and mild PKU plasma BH₄ levels may be not high enough to fully activate the liver phenylalanine hydroxylase and thus lower blood phenylalanine levels. Assessment of plasma BH₄ or total biopterin concentrations may be a good way to control the efficacy of the loading test.

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Introduction

Besides its function as a cofactor for aromatic amino acid hydroxylases, nitric oxide synthase, and glycerol-ether monooxygenase (Fig. 1) [1], tetrahydrobiopterin (BH₄) is of great importance as a pharmacological compound in the treatment of patients with different

forms of hyperphenylalaninemia (HPA) [2]. Both, patients with defects in the biosynthesis of BH₄ [2] as well as patients with BH₄-responsive phenylalanine hydroxylase deficiency (PAH) [3,4], benefit from substitution with the synthetic cofactor. The commercially available active form of BH₄ (6R-BH₄) is usually administered orally at doses of 2–10 mg/kg body weight in order to maintain the normal hydroxylation of phenylalanine to tyrosine in the liver. Only very few patients with cofactor deficiency respond to BH₄ monotherapy by correcting the impaired biogenic amines homeostasis in the brain [5]. BH₄ application was further discussed in

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association with neuropsychiatric diseases such as depression [6], autism [7], Parkinsonism [8], and Alzheimer disease [9]. Due to its function as a cofactor of nitric oxide synthase, BH₄ has been investigated in cardiovascular diseases accompanied with endothelial dysfunction [10].

Another important application of BH₄ is the loading test in patients with HPA [11]. This test discriminates between BH₄-responders and non-responders, but can not differentiate between the cofactor deficiency and BH₄-responsive PAH deficiency. Additional tests such as urinary pterins and dihydropteridine reductase activity measurements are essential for an exact diagnosis [2]. Oral administration of BH₄ (20 mg/kg) reduces plasma phenylalanine concentrations in BH₄-responders (BH₄-deficient patients and patients with mild PAH deficiency): however, in contrast to patients with cofactor defects, in patients with PAH deficiency the response is not always clear [12].

Regardless of the type of application, therapeutic efficiency of BH₄ depends on factors such as the product quality and stability, dosage, mode of administration, and its pharmacokinetic properties. The aim of this study was to establish a method for the measurement of biologically active BH₄ in plasma after oral administration in adult control persons and to obtain preliminary estimates for some key pharmacokinetic parameters.

Materials and methods

Pterin compounds as well as BH₄ tablets (50 mg 6R-BH₄, 50 mg ascorbic acid, and 25 mg *N*-acetyl-cysteine) were purchased from Schircks Laboratories (Jona, Switzerland). All other chemicals were purchased from Fluka AG (Buchs, Switzerland), if not stated otherwise.

Subjects

BH₄ tablets were administered to four of us (DG, BS, BT, and NB). All subjects were healthy adult males (age 27–56 years). Blood pressure and routine clinical chemistry were monitored during the entire challenge.

BH₄ loading test

The loading test modalities are summarized in Table 1. BH₄ tablets were administered orally (dissolved in orange juice or water), at least 30 min before breakfast, at a dosage of 2, 10, or 20 mg/kg body weight.

Blood sampling

Blood was collected in 2.7 mL EDTA tubes (Sarstedt, Sevelen, Switzerland) containing either 0.1% (w/v) dithioerythritol (DTE), 0.04% (w/v) ascorbic acid, or without antioxidant, immediately centrifuged at 2000g for 10 min, and stored at –80 °C.

Iodine oxidation

Total biopterin represents the sum of BH₄, 7,8-dihydrobiopterin, and fully oxidized biopterin. Differential oxidation with iodine according to Fukushima and Nixon [13] enables measurement of both total biopterin and BH₄. Under acidic conditions BH₄ and 7,8-dihydrobiopterin are oxidized to biopterin, while under basic conditions only 7,8-dihydrobiopterin is oxidized to biopterin and BH₄ undergoes side-chain cleavage to form pterin. The difference in biopterin content between the two oxidations represents the actual BH₄ levels.

Acidic pH oxidation

Ninety microliters of plasma and 10 µL of the internal standard (rhamnopterin 400 nmol/L) were acidified by addition of 20 µL of 1 M hydrochloric acid and 50 µL of iodine solution (1% (w/v) iodine in 2% (w/v) potassium iodide) were added. Samples were mixed and incubated for 1 h in the dark at room temperature. The reaction was stopped by adding 10 µL of 5% (w/v) ascorbic acid and 20 µL water.

Basic pH oxidation

To the same volume of plasma and internal standard 20 µL of 1 M sodium hydroxide were added and oxidation was performed as described above. Samples were mixed and incubated for 1 h in the dark at room temperature. The reaction was stopped by adding 10 µL of

Table 1
Amount of orally administered BH₄ and time intervals of the blood sampling

| Trial no. | Subject | BH ₄ mg/kg (total amount) | Blood sampling (hours) |
|-----------|-----------------|--------------------------------------|---------------------------------|
| 1 | BS ^a | 10 mg/kg (900 mg) | 0, 1, 2, 3, 4, 6, 8, 10 |
| 2 | BS ^a | 20 mg/kg (1800 mg) | 0, 1, 2, 3, 4, 6, 8, 10 |
| 3 | BT | 10 mg/kg (700 mg) | 0, 1, 2, 3, 4, 6, 8, 10, 24, 33 |
| 4 | DG | 10 mg/kg (850 mg) | 0, 1, 2, 3, 4, 6, 10, 24 |
| 5 | NB | 2 mg/kg (150 mg) | 0, 1, 3 |
| 6 | NB ^b | 2 mg/kg (150 mg) | 0, 0.5, 1, 2, 3 |

^a Samples were analyzed after 8 months at –80 °C.

^b Sublingual application.

5% (w/v) ascorbic acid and 20 μ l of 2 M hydrochloric acid.

Oxidized samples were filtered in Millipore ultrafilter with 10,000 MW cut-off (Millipore, Bedford, MA) by centrifugation at 5000g for 30 min. The clear filtrate was injected into the HPLC system (see below).

HPLC of pterins

HPLC of pterins (neopterin, biopterin, isoxanthopterin, pterin, and rhamnopterin) was performed as described previously [14] with some modifications. Separation was performed on a C8 Spherisorb, 5 μ m pre-column (40 \times 4.6 mm) and ODS-1 Spherisorb, 5 μ m analytical column (250 \times 4.6 mm) (both from Stagroma, Rheinach, Switzerland), using 1.5 mmol/L potassium hydrogen phosphate buffer, pH 4.6, with 8% (v/v) methanol at a flow rate of 1.2 mL/min. Pterins were detected by their native fluorescence at λ_{EX} : 350 nm, λ_{EM} : 450 nm using a fluorescence Detector FP-920 (Jasco, Tokyo, Japan).

Pharmacokinetic

Pharmacokinetic parameters were calculated using the PK Solutions software, v. 2.0 (Summit Research Services, Montrose, CO).

Results

Analysis and stability of BH₄ in blood

In order to determine the optimal condition for the handling and storage of samples, blood was collected after BH₄ administration (10 mg/kg) in the presence of either 0.1% DTE or 0.04% ascorbic acid, and plasma BH₄ and total biopterin concentrations were compared with those of blood samples collected without antioxidants after storage at -80°C for 8 months. Fig. 2 shows that pretreatment of blood with DTE or ascorbic acid prevents oxidation of BH₄. Determination of BH₄ in plasma was not possible without antioxidant stabilization and BH₄ was more stable with addition of DTE than with ascorbic acid, when analysis was performed after a longer period of storage (8 months) (Fig. 2A). Compared with DTE plasma, total biopterin concentrations were on average 61 and 64% lower then when preserved with ascorbic acid or without antioxidant, respectively (Fig. 2B). Although ascorbic acid prevents BH₄ oxidation, only DTE protects from both oxidation and side-chain cleavage to pterin (Fig. 3C). When analysis is performed immediately after blood sampling, 64.8–90.3% (mean 80.7%) of total biopterin is present as BH₄, regardless whether ascorbic acid or DTE are used as an antioxidants (Fig. 3). Using higher concentrations

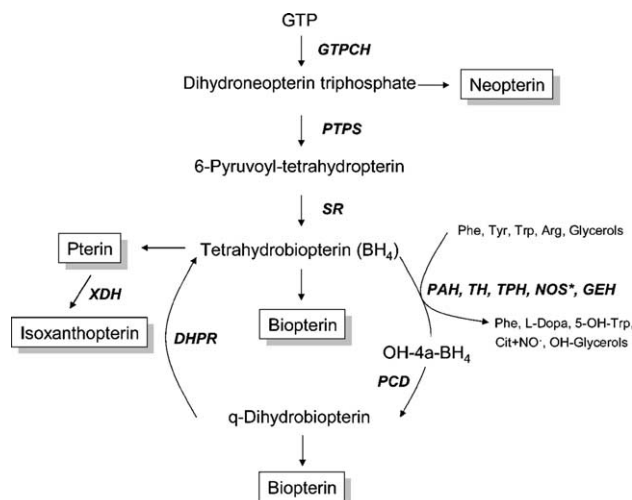


Fig. 1. Tetrahydrobiopterin metabolism and functions. GTPCH, GTP cyclohydrolase I; PTPS, 6-pyruvoyl-tetrahydropterin synthase; SR, sepiapterin reductase; PCD, pterin-4a-carbinolanin dehydratase; DHPD, dihydropteridine reductase; PAH, phenylalanine-4-hydroxylase; TH, tyrosine-3-hydroxylase; TPH, tryptophan-5-hydroxylase; NOS, nitric oxide synthase; GEH, glycerol-ether monooxygenase; and XDH, xanthin dehydrogenase. *Note that NOS reaction does not require the two regenerating enzymes PCD and DHPD.

of either DTE or ascorbic acid did not enhance the stability of BH₄ in blood (data not shown).

Pharmacokinetic

Preliminary pharmacokinetic parameters for BH₄ were estimated after oral administration of 10 mg/kg to three different subjects (BS, BT, and DG) with different sampling times (Table 2). One subject (BS) was loaded with 10 and 20 mg/kg. Because of BH₄ stability problems (see above) the total biopterin values were used for the calculation of pharmacokinetic parameters. Maximal plasma biopterin levels peaked between 1 and 4 h (C_{max} = 258.7–295.0 nmol/L for 10 mg/kg) and 24 h after administration BH₄ levels were still 4–5 times the basal values, showing a first order kinetics (Fig. 4). The plasma concentration curve shows a fast absorption phase (T_0 – T_4 h), a rapid fall (T_4 – T_{10} h) corresponding to the absorption and distribution phase followed by a slower decline in the terminal elimination phase (T_{10} – T_{33} h). The area under the curve (AUC) up to 10 h after a dose of 10 mg/kg amounted to 1708–1958 nmol \cdot h/L (3 subjects). The AUC up to 24 h amounted to 2473 and 2974 nmol \cdot h/L (2 subjects) and AUC up to 33 h to 2956 nmol \cdot h/L (1 subject). Therefore, the apparent clearance (CL/F) was estimated to 900 L/h. Extrapolated $AUC_{0-\infty}$ was between 2959 and 3603.

Subject BS administered BH₄ at two different doses (10 and 20 mg/kg). Maximal plasma biopterin concentrations were reached after 3 and 4 h, respectively (Fig. 5); the elimination kinetics seem to be only slightly

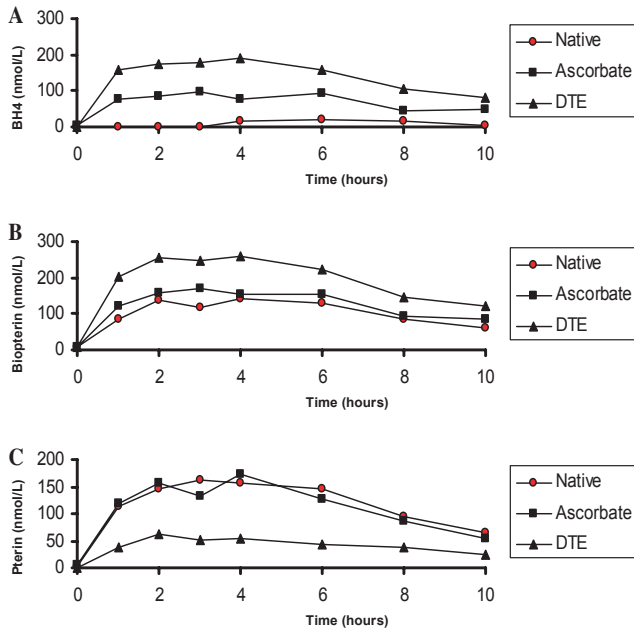


Fig. 2. Concentrations of (A) BH₄, (B) total biopterin, and (C) pterin after oral administration of BH₄ (10 mg/kg) in plasma without any additive (●), pretreated with 0.04% ascorbic acid (■), or with 0.1% DTE (▲) in subject BS.

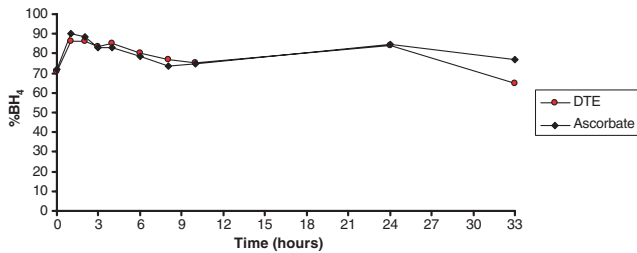


Fig. 3. Percentage of BH₄ of the sum of biopterin, dihydrobiopterin, and BH₄ in plasma pretreated with 0.04% ascorbic acid (◆) or 0.1% DTE (●) in subject BT following oral administration of BH₄ (10 mg/kg).

faster at higher plasma concentrations ($T_{1/2}$: 4.2 vs. 5.1 h; $T = 4-10$ h). 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).

In order to investigate the effect of repeated BH₄ administrations and to compare oral with sublingual administration, BH₄ was given orally to subject NB on

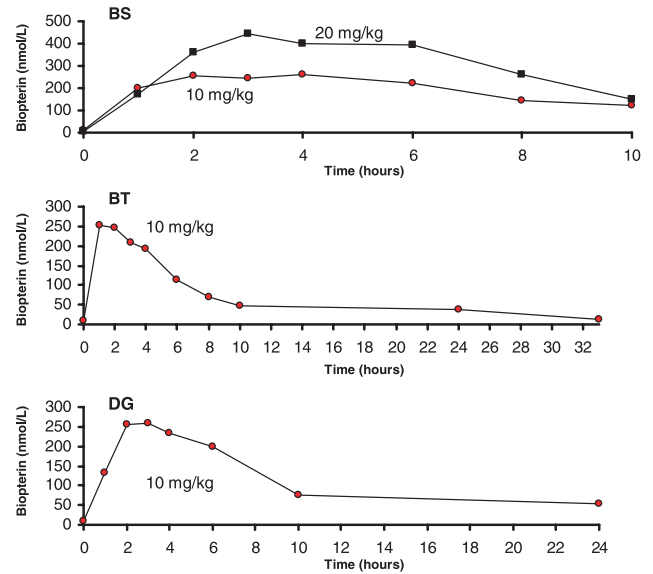


Fig. 4. Total plasma biopterin profiles from subjects BS, BT, and DG following oral administration of BH₄ (10 or 20 mg/kg).

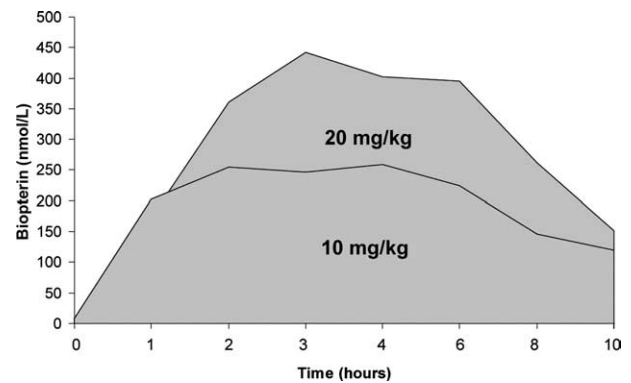


Fig. 5. Total plasma biopterin levels before, and 1 and 3 h after repeated administration of BH₄ (2 mg/kg) as a single dose on 3 consecutive days in subject NB.

three consecutive days at a dosage of 2 mg/kg. Both basal and 3-h plasma biopterin levels increased by an average of 15% on days 2 and 3 (Fig. 6A). After withdrawal for 1 week the same amount of BH₄ was administered sublingually to the same person. The problem with sublingual application was the rather acidic taste of three 50 mg tablets and increased salivation. Thus, due to swallowing of some BH₄ with saliva

Table 2
Summary of pharmacokinetic parameters

| Trial no. | Dosage (mg/kg) | T_{max} (h) | C_{max} (nmol/L) | $T_{1/2}$ (h) | AUC_{0-10} (nmol*h/L) | AUC_{0-24} (nmol*h/L) | AUC_{0-33} (nmol*h/L) | $AUC_{0-\infty}$ (nmol*h/L) |
|-----------|----------------|---------------|--------------------|---------------|-------------------------|-------------------------|-------------------------|-----------------------------|
| 1 | 10 | 4 | 258.7 | 5.1 | 1958 | – | – | 3159 |
| 2 | 20 | 3 | 441.7 | 4.2 | 3046 | – | – | 3603 |
| 3 | 10 | 1 | 295.0 | 3.3 | 1708 | 2473 | 2760 | 2959 |
| 4 | 10 | 3 | 286.7 | 3.6 | 1858 | 2974 | – | 3279 |

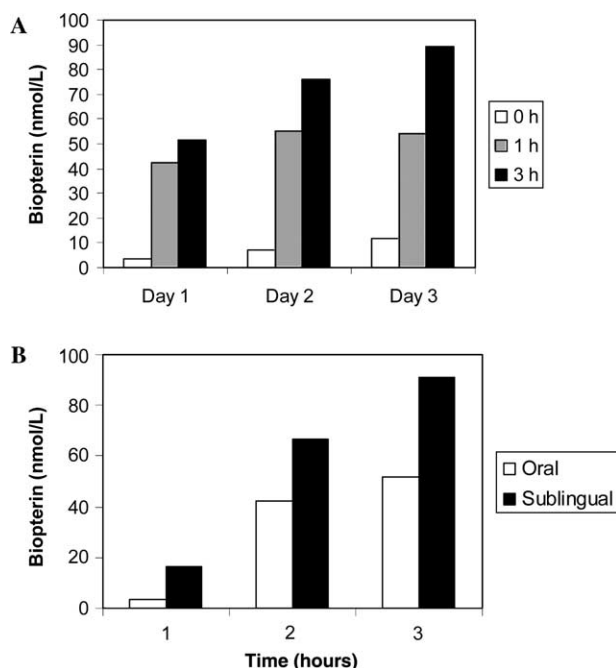


Fig. 6. Total plasma biopterin levels after oral and sublingual administration of BH₄ (2 mg/kg) in subject NB. (A) Biopterin concentration on days 1–3, before and 1 and 3 h after sublingual administration of BH₄. (B) Comparison of biopterin concentrations 1, 2, and 3 h after oral or sublingual administration of BH₄.

this experiment should be judged as partially sublingual. Nevertheless, sublingual application resulted in 58 and 76% higher plasma biopterin levels 1 and 3 h after administration, respectively, compared with oral administration (Fig. 6B).

Other laboratory tests

Routine clinical chemistry including urea, creatinine, transaminase, creatine kinase, and amino acids in plasma showed no alteration during the entire loading test (data not shown). There was no change in blood pressure when loading with 10 mg/kg; however, increasing the dosage to 20 mg/kg resulted in a transient decrease of both systolic and diastolic blood pressure (~10% reduction) in subject BS (data not shown).

Discussion

With the growing application of BH₄ in medicine there is a need for the characterization of its pharmacokinetic properties and thus for a reliable method for the measurement of both BH₄ and total biopterin in plasma. BH₄ is currently being used as a potential pharmacological agent in three major areas of clinical research. The best established and also the best defined group comprises patients with BH₄ deficiency [2] and those with the recently recognized BH₄-responsive PAH

deficiency [3,15]. Most patients with BH₄ deficiency and about 60% of mild HPA/PKU patients can be potentially treated with BH₄. In the second group are patients with psychiatric disorders, including autistic children. However, only a few of these patients showed so far clear benefits from BH₄ substitution [7,16,17]. The third and rapidly growing group covers patients with cardiovascular problems due to endothelial dysfunction [18]. It has been shown that BH₄ plays an important role in restoring the impaired endothelial function and vasoactivity by regulating NOS [19–21]. Since BH₄ oxidation leads to uncoupling of endothelial NOS, measurement of plasma BH₄ and not total biopterin is particularly important in this group of patients.

The method of Fukushima and Nixon [13] is widely used for the measurement of BH₄ in different body fluids. We modified this method by replacing the ion-exchange pretreatment of samples with a HPLC system with column switching [14] and we protected the BH₄ in blood by pretreatment with antioxidants. Both, DTE and ascorbic acid were tested as antioxidants and we found slightly better results with 0.1% DTE. Without addition of antioxidants to the blood, BH₄ was rapidly oxidized to dihydrobiopterin and biopterin and to some extent it was converted non-enzymatically to pterin, probably by side-chain cleavage. Even when pretreated with DTE only about 80% of total biopterin was found to be present in tetrahydroform. In contrast to cardiovascular disorders where the concentrations of BH₄ in plasma are of primary interest, for pharmacokinetic studies and therapy monitoring in patients with HPA total biopterin concentrations seems to be informative enough. Also, it is rather difficult to manage strictly standardized blood collection during the BH₄ loading test in newborns and some hospitals do not have ready access to laboratory facilities. Thus, assuming that almost all BH₄ in the circulating blood is present in tetrahydroform, we used the total biopterin for calculating the pharmacokinetic parameters.

Very little is known about BH₄ pharmacokinetics in humans. It has been shown that only a small portion of orally administered BH₄ is excreted in urine as biopterin or converted to lumazines in the gut [22,23]. We assume that most of the ingested BH₄ is used as a cofactor (mainly for PAH in liver) and catabolized to non-fluorescing compounds; possibly it is even degraded to CO₂ and ammonia. After intravenous injection of low dose [¹⁴C]BH₄ (45 µg/kg) in mice, high levels of radioactivity were found to accumulate in the liver and kidney and very little was found in the brain, adrenal medulla, and bone marrow [24]. In contrast to BH₄ oxidized biopterin was not accumulated in any tissue and was rapidly excreted. The BH₄ metabolism and disposition was most extensively studied in rats by Hayashi et al. [25]. The authors showed that 2-h plasma concentrations of total biopterin depend on the route of BH₄ administration

with the highest absorption after oral administration of 100 mg/kg BH₄ found in the duodenum (~14 nmol/L), followed by the jejunum (~6 nmol/L), and with only minimal absorption in the stomach. Furthermore, intestinal absorption of BH₄ seems to be age-dependent. Hayashi et al. measured 2-h plasma bipterin concentrations after oral administration of 10 mg/kg BH₄ to rats between 4 days and 8 weeks after birth and found 5-times higher levels in young rats (age 4 days–2 weeks) compared with older animals (3–8 weeks). They also compared BH₄ distribution after administration of [³H]BH₄ by whole-body radiography and found 30 min after intravenous administration most of the radioactivity in the liver and kidney. Some intestinal radioactivity was still found 6 h after intravenous administration. In contrast, 2–6 h after oral administration of labeled BH₄ most of the radioactivity was found in the gastrointestinal tract. Intraperitoneal administration showed accumulation of BH₄ in different organs including the liver and kidney, but also in heart and lung, and 6 h after injection most of the radioactivity was found in the urinary bladder. Again, distribution was age-dependent, with higher concentrations in younger animals. They calculated the half-life time for orally administered BH₄ and found it to be 1 and 3 h for the 6 and 2 weeks old mice, respectively. However, one should take into account that these parameters were calculated from the 3–6 h slope. Another interesting observation is that BH₄ crosses the blood–brain barrier more efficiently in younger mice. Oral administration of BH₄ (10 mg/kg) resulted in 5-times greater AUC for the brain in 2 weeks old mice compared with higher dosage (100 mg/kg) in 6 weeks old mice.

Our results suggest rather large variability of orally administered BH₄, probably due to different absorption in the gut and/or to the first-passage effect (Table 2). Repeated administration of low-dosage BH₄ (2 mg/kg) resulted in increasing plasma BH₄ concentrations. The most interesting finding is that administration of BH₄ by the sublingual route resulted in about 60% higher plasma BH₄ concentrations (Fig. 6B). This despite the fact that relative large tablets (three 50 mg tablets for a dosage of 2 mg/kg) and a rather unpleasant taste (acidic due to hydrochloride formulation and ascorbic acid) in this experiment resulted in probably not 100% sublingual absorption. Thus, designing a more “friendly” formulation of BH₄ with better taste and a higher amount of BH₄ (100 or 200 mg) may be an interesting alternative to the classical oral application. Such tablets could be used only in older children and adults, however, in significantly lower doses than now recommended, and thus resulting in much lower cost of the treatment. Current costs for BH₄ are rather high with the estimated US\$13,000 per year for a 7 years old child.

Based on present knowledge the response of HPA patients to oral administration of BH₄ depends not only

on the mutation in the PAH gene, but also on initial phenylalanine levels, dose and quality of BH₄ administered, and its pharmacokinetics properties for each person. The individual plasma BH₄ (or total bipterin) profile seems to be important for the interpretation of a BH₄ loading test. The differences in pharmacokinetic parameters we described between different subjects may explain the variable responses to the BH₄ loading test in patients with the same PAH genotype. One can assume that in some patients with expected BH₄-responsiveness (known PAH mutations) the effect of BH₄ was not evident because of probably poor intestinal absorption. This assumption is supported by the analysis of urinary pterins in patients with HPA after oral administration of BH₄ (20 mg/kg) showing total bipterin concentrations in urine ranging between 2.0 and 60.1 mmol/mol creatinine (median = 22.2 mmol/mol; *n* = 50).

Based on the elimination half-life time of about 4 h we suggest that in patients with BH₄ deficiency and BH₄-responsive HPA/PKU, BH₄ should be administered in at least 2–3 daily doses in order to optimize the therapeutic response.

Oral administration of up to 20 mg/kg BH₄ did not affect routine clinical chemistry parameters and except for subject DG, no adverse effect was experienced. In subject DG abdominal pains that occurred about 18 h after administration of 10 mg/kg BH₄ may be not directly related to BH₄. Interestingly, higher doses of BH₄ (20 mg/kg) reduced both systolic and diastolic blood pressure in subject BS, who was not hypertensive, by 10%. These data may fit well with previous observations that BH₄ supplementation normalizes blood pressure in hypertensive rats [26] and that downregulation of BH₄ synthesis contributes to increased blood pressure in glucocorticoid hypertensive rats [27].

Further investigation of the BH₄ loading test as well as inter- and intra-individual variations in patients with BH₄-responsive HPA should help in characterizing particular phenotypes and may provide additional information for the interpretation of phenotype–genotype correlation. It is possible that pharmacokinetics of BH₄ in disease are different from what is known in healthy subjects. Measurement of plasma BH₄ or total bipterin levels is an important tool to control the efficiency of each loading test.

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