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 \( T_{\text{max}} \) (1–4 h), \( C_{\text{max}} \) (258.7–259.0 nmol/L biopterin at a dosage of 10 mg/kg), and area under the curve (AUC = 1708–1958 nmol/C211/C212h/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 monoxygenase (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 cannot 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 2000 g 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>
<thead>
<tr>
<th>Trial no.</th>
<th>Subject</th>
<th>BH₄ mg/kg (total amount)</th>
<th>Blood sampling (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BSa</td>
<td>10 mg/kg (900 mg)</td>
<td>0, 1, 2, 3, 4, 6, 8, 10</td>
</tr>
<tr>
<td>2</td>
<td>BSa</td>
<td>20 mg/kg (1800 mg)</td>
<td>0, 1, 2, 3, 4, 6, 8, 10</td>
</tr>
<tr>
<td>3</td>
<td>BT</td>
<td>10 mg/kg (700 mg)</td>
<td>0, 1, 2, 3, 4, 6, 8, 10, 24, 33</td>
</tr>
<tr>
<td>4</td>
<td>DG</td>
<td>10 mg/kg (850 mg)</td>
<td>0, 1, 2, 3, 4, 6, 10, 24</td>
</tr>
<tr>
<td>5</td>
<td>NB</td>
<td>2 mg/kg (150 mg)</td>
<td>0, 1, 3</td>
</tr>
<tr>
<td>6</td>
<td>NBb</td>
<td>2 mg/kg (150 mg)</td>
<td>0, 0.5, 1, 2, 3</td>
</tr>
</tbody>
</table>

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

b Sublingual application.
5% (w/v) ascorbic acid and 20 μl of 2 M hydrochloric acid.

Oxidized samples were filtered in Millipore ultrafilter with 10,000 MW cut-off (Millipore, Bedford, MA) by centrifugation at 5000×g 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 μm pre-column (40 × 4.6 mm) and ODS-1 Spherisorb, 5 μm analytical column (250 × 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<sub>EX</sub>: 350 nm, λ<sub>EM</sub>: 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 BH<sub>4</sub> in blood**

In order to determine the optimal condition for the handling and storage of samples, blood was collected after BH<sub>4</sub> administration (10 mg/kg) in the presence of either 0.1% DTE or 0.04% ascorbic acid, and plasma BH<sub>4</sub> 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 BH<sub>4</sub>. Determination of BH<sub>4</sub> in plasma was not possible without antioxidant stabilization and BH<sub>4</sub> 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 BH<sub>4</sub> 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 BH<sub>4</sub>, regardless whether ascorbic acid or DTE are used as antioxidants (Fig. 3). Using higher concentrations of either DTE or ascorbic acid did not enhance the stability of BH<sub>4</sub> in blood (data not shown).

**Pharmacokinetic**

Preliminary pharmacokinetic parameters for BH<sub>4</sub> 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 BH<sub>4</sub> 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 (<i>C<sub>max</sub></i> = 258.7–295.0 nmol/L for 10 mg/kg) and 24 h after administration BH<sub>4</sub> 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 (<i>T<sub>0</sub></i>–<i>T<sub>4</sub></i> h), a rapid fall (<i>T<sub>4</sub></i>–<i>T<sub>10</sub></i> h) corresponding to the absorption and distribution phase followed by a slower decline in the terminal elimination phase (<i>T<sub>10</sub></i>–<i>T<sub>33</sub></i> h). The area under the curve (AUC) up to 10 h after a dose of 10 mg/kg amounted to 1708–1958 nmol·h/L (3 subjects). The AUC up to 24 h amounted to 2473 and 2974 nmol·h/L (2 subjects) and AUC up to 33 h to 2956 nmol·h/L (1 subject). Therefore, the apparent clearance (CL/F) was estimated to 900 L/h. Extrapolated AUC<sub>0−</sub><sub><i>∞</i></sub> was between 2959 and 3603.

Subject BS administered BH<sub>4</sub> 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
faster at higher plasma concentrations ($T_{1/2} = 2: 4.2$ vs. $5.1$ 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/L).

In order to investigate the effect of repeated BH$_4$ administrations and to compare oral with sublingual administration, BH$_4$ was given orally to subject NB on 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 BH$_4$ 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 BH$_4$ with saliva

<table>
<thead>
<tr>
<th>Trial no.</th>
<th>Dosage (mg/kg)</th>
<th>$T_{max}$ (h)</th>
<th>$C_{max}$ (nmol/L)</th>
<th>$T_{1/2}$ (h)</th>
<th>AUC$_{0-10}$ (nmol·h/L)</th>
<th>AUC$_{0-24}$ (nmol·h/L)</th>
<th>AUC$_{0-33}$ (nmol·h/L)</th>
<th>AUC$_{0-1}$ (nmol·h/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>4</td>
<td>258.7</td>
<td>5.1</td>
<td>1958</td>
<td>–</td>
<td>–</td>
<td>3159</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>3</td>
<td>441.7</td>
<td>4.2</td>
<td>3046</td>
<td>–</td>
<td>–</td>
<td>3603</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>1</td>
<td>295.0</td>
<td>3.3</td>
<td>1708</td>
<td>2473</td>
<td>2760</td>
<td>2959</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>3</td>
<td>286.7</td>
<td>3.6</td>
<td>1858</td>
<td>2974</td>
<td>–</td>
<td>3279</td>
</tr>
</tbody>
</table>
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 extend 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 biopterin 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 biopterin) 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 biopterin 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 biopterin levels is an important tool to control the efficiency of each loading test.

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