RESEARCH ARTICLE

Tetrahydrobiopterin Supplementation: Elevation of Tissue Biopterin Levels Accompanied by a Relative Increase in Dihydrobiopterin in the Blood and the Role of Probenecid-Sensitive Uptake in Scavenging Dihydrobiopterin in the Liver and Kidney of Rats

Akiko Ohashi1,*, Yusuke Saeki2, Tomonori Harada3, Masako Naito1, Tomihisa Takahashi1, Shin Aizawa3, Hiroyuki Hasegawa1,3

1 Department of Anatomy, Nihon University School of Dentistry, Chiyoda, Tokyo, Japan, 2 Department of Biosciences, Teikyo University of Science and TechnologyUenohara, Yamanashi, Japan, 3 Division of Anatomical Science, Department of Functional Morphology, Nihon University School of Medicine, Itabashi, Tokyo, Japan

* oohashi.akiko@nihon-u.ac.jp

Abstract

Tetrahydrobiopterin (BH₄) is an essential cofactor of nitric oxide synthase (NOS) and aromatic amino acid hydroxylases. BH₄ and 7,8-dihydrobiopterin (BH₂) are metabolically inter-changeable at the expense of NADPH. Exogenously administered BH₄ can be metabolized by the body, similar to vitamins. At present, synthetic BH₄ is used as an orphan drug for patients with inherited diseases requiring BH₄ supplementation. BH₄ supplementation has also drawn attention as a means of treating certain cardiovascular symptoms, however, its application in human patients remains limited. Here, we tracked biopterin (BP) distribution in blood, bile, urine, liver, kidney and brain after BH₄ administration (5 mg/kg rat, i.v.) with or without prior treatment with probenecid, a potent inhibitor of uptake transporters particularly including organic anion transporter families such as OTA1 and OAT3. The rapid excretion of BP in urine was driven by elevated blood concentrations and its elimination reached about 90% within 120 min. In the very early period, BP was taken up by the liver and kidney and gradually released back to the blood. BH₄ administration caused a considerable decrease in the BH₄% in blood BP as an inevitable compensatory process. Probenecid treatment slowed down the decrease in blood BP and simultaneously inhibited its initial rapid excretion in the kidney. At the same time, the BH₄% was further lowered, suggesting that the probenecid-sensitive BP uptake played a crucial role in BH₂ scavenging in vivo. This suggested that the overproduced BH₂ was taken up by organs by means of the probenecid-sensitive process, and was then scavenged by counter-conversion to BH₄ via the
BH₄ salvage pathway. Taken together, BH₄ administration was effective at raising BP levels in organs over the course of hours but with extremely low efficiency. Since a high BH₂ relative to BH₄ causes NOS dysfunction, the lowering of the BH₄% must be avoided in practice, otherwise the desired effect of the supplementation in ameliorating NOS dysfunction would be spoiled.

Introduction

Tetrahydrobiopterin (BH₄) is an essential cofactor for a group of aromatic amino acid hydroxylases [1–4]. Inherited BH₄ deficiencies are characterized by hyperphenylalaninemia and defective biosynthesis of classic monoamines such as dopamine, noradrenaline, adrenaline, as well as serotonin. BH₄ therapy using (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin dihydrochloride (6RBH₄) has been very successful in replacing peripheral BH₄, but not BH₄ in the brain. A guide for the therapeutic use of BH₄ is available [5]. This compound ameliorates hyperphenylalaninemia in cases of inherited BH₄ deficiency [6, 7] and benefits patients with BH₄-responsive phenylketonuria [8]. The most common use of 6RBH₄ to date is as an orphan drug for these inherited diseases [9–11]. Once 6RBH₄ is administered, it replaces innate BH₄ and is integrated through endogenous metabolic pathways, similar to the intake of vitamins. However, like most drugs or supplements, large amounts of exogenous BH₄ bypass endogenous pathways and are eliminated in urine [12, 13] and feces [14], therefore it is desirable to minimize this useless exclusion.

We previously observed that 6RBH₄ administration raised tissue BH₄ levels, however, with a concomitant rise in 7,8-dihydrobiopterin (BH₂) [12, 13]. Urinary excretion was suggested to be the process most responsible for the rapid loss of exogenous BH₄. Furthermore, we demonstrated that urinary excretion of administered 6RBH₄ at a pharmacological dose predominantly involved secretion across renal tubular epithelium, distinct from glomerular filtration. We observed that the tubular secretion was almost completely suppressed by cyclosporin A (CsA), an inhibitor of transporters with broad specificity in excretion of xenobiotics and metabolic wastes.

In order to obtain greater insight into the renal exclusion of BH₄ and the relative increase in dihydrobiopterin (BH₂), in this study, we analyzed the systemic distribution of biopterin (BP), the sum total of BH₄ and BH₂ (BP = BH₂ + BH₄), in the presence of probenecid (Pbc), a potent inhibitor of uptake transporters with a broad specificity [15]. We confirmed the active role played by the renal secretion of BP in the heavy loss of exogenous BH₄. Furthermore, we revised the role of the salvage pathway of BH₄ biosynthesis in scavenging BH₂ in terms of its passage through the liver and kidney under the conditions of 6RBH₄ administration.

Materials and Methods

Chemicals

6RBH₄ was donated by Suntory (Asubio Pharma, Kobe, Japan). 6RBH₄ (BH₄•2HCl) was dissolved at 10 mg/mL in 0.9% NaCl before the start of the BH₄ administration experiment. The BH₄ solution was neutralized by 0.1 M Na₂HPO₄ and saline to prepare a 5 mg/mL concentration immediately before injection. Probencid (Pbc: p-(dipropylsulfamoyl)benzoic acid) and cyclosporin A were obtained from Sigma-Aldrich (St. Louis, MO). Pbc was dissolved by dropwise addition of 2M NaOH, followed by the addition of N-2-hydroxyethylpiperazine-N’-2’-
ethanesulfonic acid (HEPES) to neutralize the solution and adjusted the concentration to 40 mg/mL. CsA was dissolved in ethanol, then diluted in 9 vol of olive oil (final concentration, 5 mg/mL). “Gall Powder” was purchased from Wako Pure Chemical Industries (Osaka, Japan).

**Ethics statement**

The animal experiments were conducted in accordance with the ethical guidelines of the Teikyo University of Science and Technology Animal Experimentation Committee, and guidelines of the Japanese Pharmacological Society. The protocol was approved by the Teikyo University of Science and Technology Animal Experimentation Committee (Permit Number: B-08005). All efforts were made to minimize suffering. Rats (SD: Sprague Dawley) were obtained from Japan SLC (Hamamatsu, Japan) and bred in our SPF-grade laboratory. The animals were maintained on a constant 12-h light-dark cycle at 21–24°C at 40–60% humidity and were provided with ordinary laboratory chow and sterile tap water ad libitum. For deep but a short period of anesthesia, rats were administered sodium pentobarbital (30 mg/kg for light anesthesia, 50 mg/kg for deep, both i.p.). For experiments taking a longer time (4 ~ 6 hours), rats were anesthetized with an i.p.-administered cocktail of pentobarbital (45 mg/kg), atropine (0.05 mg/kg), dimorpholamine (Theraptique, 1.2 mg/kg), and xylazine (9.5 mg/kg) as described previously [12, 13]. The anesthetized rats were kept on a warm gel pad (40°C) and given a 1/3 to 1/2 dose every hour to maintain them under anesthesia.

**Experimental procedure**

All rats (8–10 weeks old, male and female random mix) were loaded with 6RBH$_4$ (5 mg/mL) at a dose of 5 mg/kg (i.v.) under anesthesia. Administration of Pbc (200 mg/kg, i.p.) or CsA (10 mg/kg, i.p.) was performed 30 min prior to 6RBH$_4$ administration. Experiments were carried out by means of two different protocols; (A) by sequentially collecting blood, bile and urine from individual rats under long-lasting anesthesia on a warm gel pad, and (B) by removing the liver, kidney and brain after sacrificing the respective rats under deep anesthesia at specified times after 6RBH$_4$ administration.

(A) Rats were intraperitoneally administered Pbc (“BH$_4$ + Pbc”, n = 5) or saline as a control (“BH$_4$ alone”, n = 7) and were then anesthetized. All rats were given 6RBH$_4$, then subjected to sampling at specified times as previously described [12, 13], except that bile was also taken and the animal was given saline containing 2% bovine bile powder (w/v), at a rate of 0.9 mL/hr, which was administered into the intestinal duct. The bovine bile powder did not contain any measurable BH$_2$ or BH$_4$. In the following sample acquisition, special care to ensure minimal oxidation of BH$_4$ was taken throughout the following procedure. In brief, the abdomen was opened and cannulae were set in place for collecting bile and urine. The blood samples were drawn from the tail vein into a heparin-coated capillary containing a solution of 0.1 M ascorbic acid (less than 5% by volume), and they were then subjected to differential iodine oxidation for BP determination. The urine was collected at intervals from the bladder through an indwelling needle connected to an airtight syringe in which the dead space had been filled with saline containing 50 mM each of ascorbic acid and EDTA (pH 6.8). The urine was immediately mixed with 9 volumes of 10 mM HCl containing 5 mM antioxidant. The aliquots were then subjected to quantitation of BP and creatinine. BP excretion was expressed as BP/creatinine (μmol/mg creatinine) based on the BP concentration (nmol/mL) divided by the time-matched creatinine level (μg/mL). In calculating the cumulative total, the amounts of BP in each fraction were added. Calculations were based on the BP concentration (nmol/mL) multiplied by the respective urine volume (mL) produced between each sampling time point regardless of the
creatinine concentration. The bile was also collected from individual rats through a cannula in the bile duct and was immediately subjected to BP determination. The cumulative BP amount was calculated by adding up all samples measured.

(B) Rats were treated with 6RBH₄ and drugs (Pbc and CsA), or saline as a vehicle control, and allowed to move freely after recovery from the light anesthesia. In the Pbc experiment, animal used numbered n = 4 each for the respective administration of "BH₄ + Pbc", and the vehicle control "BH₄ alone". In the CsA experiment, the numbers of rats for groups of "BH₄ + CsA" and "BH₄ alone" were n = 5, respectively. At 0, 30 and 120 min after 6RBH₄ administration, blood samples were taken from the abdominal vein into a syringe in which the dead space was replaced with saline containing 50 mM each of ascorbic acid and EDTA (pH 6.8). Each blood sample was divided into three portions; one served as a "whole blood" specimen, the second was for the hematocrit determination, and the third was centrifuged (10,000 rpm, 4°C, 5 min) to separate red blood cells (RBCs) from the plasma. The whole blood, plasma, and RBCs were separately subjected to differential iodine oxidation for the BP determination described below. The results were expressed as the amount of BP per unit volume of the original blood sample (plasma + RBC, mL) based on the hematocrit value. The kidneys, liver, and whole brain were removed without prior perfusion, rinsed with saline, blotted, weighed, frozen in liquid nitrogen, and then stored at -80°C until BP determination. BP carryover with the blood was ignored in the liver and kidney. However, for the brain, the BP carryover was estimated using an average blood inclusion of 1.2% (v/w), which was determined by a comparison of the heme content of the blood and brain homogenates based on light absorbance at 415 nm [16].

Determinations

Biopterin was determined essentially according to Fukushima and Nixon [17] and modified as described previously [18, 19]. Animal tissues contain at least 4 chemical species of the reduced biopterin family; BH₄-4a-carbinolamine (or 4a-hydroxy-tetrahydrobiopterin), quinonoid dihydrobiopterin and 7,8-dihydrobiopterin as well as BH₄. The Fukushima and Nixon method includes tissue homogenization and extraction of pterin compounds followed by measurement of amount of fully oxidized biopterin after differential iodine oxidation under acid and alkaline conditions. With their method, the biopterin quantity remaining after the acid-oxidation was taken as the total biopterin (BP = "BH₄" + "BH₂" + "oxidized biopterin") and that which remained after the alkaline-oxidation, as 7,8-dihydrobiopterin (BH₂) plus oxidized biopterin. The amount of oxidized biopterin in extracts without the iodine oxidation was disregarded in this study since the observed amount was too small for the purpose of our work. Accordingly, in this study, the biopterin amount measured after the acid iodine oxidation was taken as the sum of "BH₄" and "BH₂" ("BP" = "BH₂" + "BH₄"), and that after the alkaline oxidation, as the amount of "BH₂". The difference was therefore taken as the "BH₄" amount. The BH₄ fraction (BH₄%; the percentage of BH₄ in BP) was calculated for each individual sample. The amounts of BH₄ and BH₂ were calculated based on molecular weights of 241 g/mol and 239 g/mol, respectively. The molecular mass of the reagent 6RBH₄•2(HCl) was 315g.

The urinary creatinine concentration was measured using a creatininase-HMMPS method according to the manufacturer’s instructions (L-type Creatinine M; Wako Pure Chemical Industries, Osaka, Japan).

Data presentation

Data were expressed as means ±S.E. The statistical significance of two groups with non-repeated determination was analyzed by Student’s t-test. The significance of difference between
determinations at different times with individual animal groups was analyzed by the Paired $t$-test. Significance of difference between three groups was analyzed by Tukey’s test. All the statistical analyses were performed with Pharmaco Basic Ver.15.0.1 (Scientist Co. Ltd., Tokyo). Using results of pterin kinetics, exponential least square approximation was performed with Excel software using Microsoft Office for Mac. For the best fit by a first-order equation, $BP_t = BP_0 \cdot e^{-kt}$, data were plotted on a semi-log scale of y-axis vs. time (min) on the linear x-axis, forming straight lines as $\ln (BP_t) = \ln (BP_0) - kt$, where $BP_0$ represents the extrapolated initial concentration of BP and $BP_t$ at time $t$ (in nmol/mL of bile or μmol/mg creatinine of urine). The slope of the plot, $k$, yields the fractional rate constant (min$^{-1}$), and gives the corresponding decay half-time, $T_{1/2} = \ln(2)/k$ (min).

**Results**

**Rat biopterin levels in the blood, urine and bile after 6RBH$_4$ administration**

We tracked BP contents and BH$_4$ fractions (BH$_4$%) in blood, urine and bile after rats were administered 6RBH$_4$ (5 mg/kg, i.v.) with or without the Pbc treatment (200 mg/kg, i.p.), denoted hereafter as “BH$_4$ + Pbc” or “BH$_4$ alone”, respectively. Experimental procedure (A) in the Methods section was used.

**Blood and urine.** Prior to treatment, the combined BP level in the blood, plasma and red blood cells was $0.57 \pm 0.08$ nmol/mL and the BH$_4$% was 90% to 95%. The BP level increased after receiving 6RBH$_4$ “BH$_4$ alone”, and gradually decreased to ca. 3.3-fold ($P<0.005$) of the initial level at 300 min (Fig 1A, lower panel). The blood BP content of the “BH$_4$ + Pbc” group was much larger than that of the “BH$_4$ alone” group. Reflecting this, the AUC$_{0-120}$ of the “BH$_4$ + Pbc” group was about 10-fold more than that of the group that received “BH$_4$ alone”; 7,580 vs. 736 (nmol/mL)•min.

In the “BH$_4$ alone” rats, the blood BH$_4$% dropped significantly within 30 min and continued to drop over the experimental period (Fig 1A, upper panel). In the “BH$_4$ + Pbc” rats, the BH$_4$% was substantially decreased but the decrease proceeded gradually; its lowest value of 79% was reached at 180 min and remained low thereafter. The difference in the BH$_4$% between the “BH$_4$ alone” and “BH$_4$ + Pbc” groups was most evident at 180 min (88% vs. 79%) ($P=0.001$).

**Urinary excretion of BP (relative to creatinine, μmol/mg Cr) after 6RBH$_4$ administration in the presence or absence of Pbc** is depicted in Fig 1B. A large amount of BP appeared soon after the 6RBH$_4$ mono-loading of “BH$_4$ alone” rats, with the peak appearing at around 30 min. On the other hand, the early excretion of BP in the “BH$_4$ + Pbc” group at 30 min was strongly suppressed to 11.7% of the amount in the “BH$_4$ alone” group ($P<0.001$) while the blood BP level of “BH$_4$ + Pbc” rats was 8.5-fold higher than of the “BH$_4$ alone” rats ($P=0.03$) at the same time point. The peak was delayed and appeared between 60 and 90 min. Since glomerular filtration might not be inhibited by Pbc, the observed decrease in BP flow at 30 min was thought to be due to a transporter-mediated process across the tubular epithelial cell layer, consistent with our previous results using CsA [12, 13]. Despite the effective inhibition of the early outflow by Pbc, the outflow appeared to have been delayed, although the gross amount was not reduced. The creatinine-based AUC$_{0-270}$ of the “BH$_4$ alone” rats was 731 (μmol/mg Cr)•min, and that of the “BH$_4$ + Pbc” rats was 608 (μmol/mg Cr)•min. The gross outflow was substantially completed by 270 min. The cumulative excretion calculated from the urine-volume-based BP reached 3,150 ±360 and 3,170 ±500 nmol in the “BH$_4$ alone” and “BH$_4$ + Pbc” groups, respectively, representing about 61% of the dose used (Fig 1C). We noted that at as early as 30 min, the BP outflow amounted to 1840 ± 250 nmol in the “BH$_4$ alone” group; about 58% of the gross outflow obtained by 270 min. Similarly, it reached 87% of the gross urinary
excretion by 120 min. Most of the remainder may have been broken down to pterin or xanthopterin. However, we have no information about the rest of the BP except for an observation with mice about BP movement in the rumen of the GI tract in our previous study [14]. We observed that a considerable amount of administered 6RBH₄ appeared in the rumen of mouse small intestine and moved to the caecum, where BP seemed not to be retrieved but essentially metabolized to pterin, a bare pterin-ring compound, by the cecal microflora.

The urinary BH₄% before receiving 6RBH₄ was 81 ± 4% (Fig 1B, upper panel). On BH₄ mono-loading, the percentage had risen to 95 ± 1% at 30 min (Paired t-test, P = 0.005), presumably representing the amount that reached the kidney bypassing uptake by various organs. Subsequently, the BH₄% was decreased to 87 ± 2% at 90 min (P = 0.01), followed by a gradual increase up to ca. 94% at 240 min (120 min vs. each data point from 150–240 min, P < 0.005). Prior to administration of 6RBH₄, but 30 min after receiving Pbc, the BH₄% was 80 ± 7%. It remained relatively low for 120 min and then rose gradually (90 min vs. each data point from 210–270 min, P < 0.005).

**Bile.** Excretion of BP in the bile was increased after administration of 6RBH₄ either with or without Pbc. Levels of the bile BP were consistently higher than BP levels of time-matched
blood samples taken from “BH₄ alone” rats (compare Fig 2A with Fig 1A). At 30 min after 6RBH₄ administration, for example, the BP level in the bile was 2.2-fold higher than that of time-matched blood (P = 0.001). The elevation of the BP content in the “BH₄ alone” group was 46% less than that of the “BH₄ alone” group at 60 min (P = 0.006). On administration of 6RBH₄, the bile BH₄% was maintained as high as 95% and was not affected by Pbc treatment over the course of the experiment (30–270 min). The bile BP profile appeared to have a limited correlation with that of the blood BP (compare upper panels in Fig 1A and Fig 2A).

Fig 2B presents kinetics based on the same data (average) of Fig 2A. The BP concentration in both groups fitted two nearly parallel lines:

“BH₄ alone”: \[BP(t) = 47.2 \times e^{-0.007t} \] (\(|R| = 0.99\) (i))

“BH₄ + Pbc”: \[BP(t) = 37.2 \times e^{-0.007t} \] (\(|R| = 0.99\) (ii))

The common rate constant of 0.007 min⁻¹ denoted a half-decay period of 99 min. This indicated that Pbc caused the BP pool size in bile to become smaller but did not change the rate constant of BP secretion, indicating that Pbc had little effect on the process. The BP pool might represent BP of the liver, the source of this fluid, suggesting that the liver supplied a smaller amount of BP in the presence of Pbc.

As shown in Fig 2C, the cumulative BP amount after administration of “BH₄ alone” for 240 min was 80.3 nmoles per rat, representing 1.6% of the 6RBH₄ dose. In addition, the cumulative BP excretion in the presence of Pbc, i.e., that of the “BH₄ + Pbc” group, was not very different;
70.8 nmoles per rat for 240 min, which was 1.4% of the 6RH4 dose. In either case, the amount of BP excreted into the bile was much less than that into the urine. We previously described excretion of BP in the bile as a route for its entero-hepatic circulation in mice, and a considerable amount of BP secreted in the small intestine was brought to the caecum [14]. In order to prevent bile BP to return to the circulation, we removed all the bile in the procedure (A). If BP was also excreted by the other route such as via the intestinal juice under the present experimental conditions, a certain amount of BP was also secreted to rumen of the small intestine and could have moved to the caecum. Possible loss of administered BP by this route remains elusive.

Organ distribution of biopterin after 6RH4 administration to rats

Organ distribution of BP was examined after 6RH4 administration (5 mg/kg, i.v.) with Pbc (200 mg/kg, i.p.) or CsA (10 mg/kg, i.p.) or without the inhibitor. In this experiment, rats were anesthetized for handling and allowed to move freely after recovery as described in experimental procedure (B).

**Blood.** The initial BP level in the plasma was 0.13 ± 0.02 nmol/mL. After rats were administered 6RH4, the plasma BP of all groups, "BH4 alone", "BH4 + Pbc" and "BH4 + CsA", was 30- to 50-fold over the initial value (Fig 3A). The elevated BP level in "BH4 + Pbc" rats at 30 min was 1.8-fold higher than that in "BH4 alone" (P<0.05). The difference between BP levels in "BH4 + CsA" and "BH4 alone" was not clear. Plasma BP levels in both groups of rats returned to around 1 nmol/mL at 120 min after 6RH4 administration but they were about 10-fold greater than the initial value. The initial BP level in RBCs was higher than in plasma (0.43 ± 0.06 nmol/mL) and roughly 77% of the blood BP was localized in these cells. The amount of BP in RBCs changed more slowly than that in plasma (Fig 3A and 3B).

![Figure 3. Effect of probenecid and cyclosporin A on the biopterin distribution in blood after 6RH4 administration.](https://example.com/figure3.png)
Increase in BP contents in the liver, kidney and brain

The BP contents increased in the liver, kidney and brain after 6RBH₄ administration as shown in Fig 4. The initial BP levels in the organs were as follows: liver, 5.86 ± 0.47; kidney, 0.68 ± 0.04; and brain, 0.41 ± 0.01 nmol/g. The protocol used was experimental procedure (B) in the Materials and Methods section.

Liver. The liver took up a large amount of BP within the short term; the BP content increased to 53.3 nmol/g (9.1-fold over the initial value) in the “BH₄ alone” rats within the first 30 min (Fig 4A). With Pbc and CsA, the effect was different; the increase in the “BH₄ + Pbc” group (5.3-fold) was considerably less than in the “BH₄ alone” group (P<0.05 at 30 min), while that of “BH₄ + CsA” was rather increased (P<0.05 at 120 min). Two interesting findings emerged: 1) the net uptake by the liver was suppressed considerably, 9.1-fold down to 5.3-fold, even though the elevation of the plasma BP doubled in the presence of Pbc (Fig 3A), suggesting that the rapid uptake was strongly inhibited to nearly one quarter by the drug. This was in sharp contrast to “BH₄ + CsA” rats in which the BP accumulation was elevated to a level corresponding to that in the blood, suggesting that CsA did not inhibit BP uptake in this organ. 2) the release process was not greatly inhibited by the drugs either Pbc or CsA; the decrease in liver BP levels in the three groups seemed proportional over the 30–120 min period. If the BP uptake was mediated by a transporter, the fact that Pbc inhibited BP uptake by the liver suggested that the putative transporter was Pbc-sensitive and was somehow inward-directional for BP transport from the blood. BP uptake by the liver in comparison to uptake by the other organs is summarized in Table 1.

Kidney. Although the endogenous BP in the kidney was only 3% of that in the liver, this organ had a relatively high capacity to uptake exogenous BP. The amount of tissue BP was greatly increased (58-fold over the initial value) in the “BH₄ alone” rats at 30 min (Fig 4B). The BP rise in the “BH₄ + Pbc” rats was even greater (150-fold), as was the rise in “BH₄ + CsA” rats (about 80-fold), both corresponding to the respective rises in the plasma BP (compare Fig 4B...
The decrease in BP in all three groups during the period between 30 and 120 min was quite rapid, similar to the change noted in plasma. Taking into account that at 30 min, the peak amount of BP in the kidney was only 4–5% of the urinary BP outflow, in which renal tubular secretion was dominant as mentioned earlier (cf. Fig 1C), it is reasonable to assume that the BP in kidney tissue transited from the plasma to the urine across the tubular epithelium. This is in remarkable contrast to the liver BP which was stored in a large quantity then released gradually into the plasma.

**Brain.** The brain BP was significantly increased in all three groups, although much less than that of the other organs (Fig 4C). The BP contents of “BH₄ alone” rats were about 43% higher after 30 min than those of untreated rats at \( P < 0.01 \). At this time point, the increase in “BH₄ + Pbc” rats was significantly larger, as much as 65% \( P < 0.01 \), compared to that of “BH₄ alone” rats \( P < 0.05 \). Since the blood content included in the brain samples was about 1.2% (v/w), it was uncertain whether the increase was caused by a blood carryover which contained increased the amount of exogenous BP (cf. Fig 3C). The BP carryover was calculated and compared with the respective BP contents of each group using mean values of BP in time-matched blood (Fig 4C). When the observed increases in BP were adjusted by subtracting the carryover, the net increase in the “BH₄ alone” rats for the first 30 min was roughly 15%. Similarly, in “BH₄ + Pbc” and in “BH₄ + CsA” rats, BP levels were elevated by 41% and 13%, respectively. Therefore, a greater increase in BP content was verified in “BH₄ + Pbc” rats than in “BH₄ alone” rats \( P < 0.05 \).

The increase in the brain BP observed in this study might not be sufficient for stimulation of monoamine biosynthesis as reported [20, 21]. In fact, Miwa et al [22] reported that a 50- to 200-fold increase in BH₄ was needed, when the preparation was injected directly into the brain ventricle for significant stimulation of monoamine biosynthesis. Although the elevation observed in this study seems to be insufficient for stimulating the biosynthesis of monoamines, it could be beneficial for neural stimulation of their release [23].

### Table 1. Biopterin distribution in organs after 6RBH₄ administration.

| organ   | organ weight (g) | relative⁴ to body | 0-time | 30 min | 120 min |
|---------|-----------------|------------------|--------|--------|---------|
|         | relative² to 250 g body weight | relative⁵ to liver | BP (nmol) | BP (nmol) | BP (nmol) |
| Liver   | 6.9             | 2.8%             | 40.4   | 367    | 268     |
|         | (100%)          | 7.1%             | 7.1%   |
| Blood   | 17              | 6.8%             | 8.32   | 76.8   | 39.5    |
|         | 21%             | 1.5%             | 0.76%  |
| Kidneys | 1.8             | 0.72%            | 1.22   | 70.6   | 17.3    |
|         | 3.0%            | 1.4%             | 0.8%   |
| Brain   | 1.5             | 0.60%            | 0.62   | 0.8    | 0.71    |
|         | 1.5%            | 0.15%            | 0.14%  |
| Sub-total| 27              | 11%              | 50.6   | 515    | 325     |
|         | n.a.            | 9.9%             | 6.3%   |

Biopterin distribution in liver compared with the other organs. Data apply to Figs 3 and 4. BP contents (nmol/organ) are expressed as the mean values and are multiplied by the respective organ weights (g).

1) Relative to a 250 g body weight, adapted from “SD-Rat Control Data 2009” of Charles River Laboratories Japan, Inc.
2) Relative to the amount of BP in whole liver (nmol)
3) Relative to dose: (increase in organ BP over 0-time) / (5180 nmol: 6RBH₄ dose per 250 g).

The BH₄ fraction (BH₄%) in organ biopterin and the effect of probenecid

Alterations in the BH₄% along with the BP distribution profiles in respective organs (Figs. 3 and 4) are depicted in Fig 5.

The BH₄% in untreated whole blood, on which the plasma and RBCs were not separated, was 96.2 ±0.2%, and it decreased significantly along with time (Fig 5A, \( P < 0.05 \)). Although the BH₄% in the plasma showed a significant but slight increase at 30 and 120 min, the lowering of
the BH₄% in RBCs was relatively large (Fig 5C, \( P < 0.01 \)) and pulled the blood BH₄% down. All samples, plasma, RBCs as well as the unseparated blood, taken from “BH₄ alone” rats showed a significantly lower BH₄% than those of “BH₄ + Pbc” rats.

The BH₄% in the liver, kidney and brain are shown in Fig 5D–5F. The initial BH₄% in these organs were: liver, 98.3 ±0.1%; kidney, 89.8 ±1.6%; and brain, 93.0±2.0%. In the liver, the BH₄% was quite stable at levels higher than 98% in all groups. In the presence of Pbc, however, namely in “BH₄ + Pbc” rats, the BH₄% was lowered slightly but significantly at 120 min (Fig 5D, “BH₄ alone” vs. “BH₄ + Pbc”, \( P < 0.01 \)). The kidney BP showed a considerable increase in its BH₄% after the 6RBH₄ administration (Fig 5E, “before” as the reference, \( P < 0.01 \)). It was noted that profiles of the BP content in the blood and kidney looked quite similar (compare Fig 3A and Fig 4B) in the period after the 6RBH₄ administration. The brain took up a small amount of blood BP as seen in Fig 4C, and no significant change in the BH₄% was observed in this study (Fig 5F). The significance of correlation between the BH₄% of blood, liver and kidney is listed in Table 2. In this Table, the BH₄% of all organs after 6RBH₄ administration with or without Pbc or CsA are listed and compared with respect to the statistical significance of differences between the organs. The BH₄% in the liver and kidney was persistently higher than that of blood BP, while the data showed no statistically significant difference between those of liver and kidney.
Discussion

Urinary excretion was the major route of BP loss while bile was a minor route.

Renal exclusion of BH₄ immediately after 6RBH₄ administration was remarkably rapid. The major portion of the exclusion, up to ca. 87% of the apparent elimination observed within 270 min, took place in the first 120 min in healthy rats. Kidney exclusion of xenobiotics and metabolic waste generally takes two paths from plasma to urine: glomerular filtration and tubular secretion [24]. The tubular secretion of exogenously administered BH₄ was effectively suppressed by prior administration of Pbc, as shown in this report, providing confirmative evidence supporting a previous observation using CsA [12, 13]. The previous report demonstrated that the urinary BP flow involved at least two processes, glomerular filtration with a rate constant of k = 0.013 min⁻¹, and tubular secretion with a 2.3-fold more rapid rate constant of k = 0.030 min⁻¹, which represented the difference between the observed gross rate constant of k = 0.043 min⁻¹ minus the glomerular rate constant. The present data on “BH₄ + Pbc” rats after 90 min (Fig 1B, lower panel) also fitted the equation BP(t) = 9.76 • e⁻⁰.⁰₁₂ᵗ (|R| = 0.92), suggesting that the urinary flow was essentially from glomerular filtration at 90 min or later after 6RBH₄ administration. The treatment with Pbc greatly increased the blood BP by inhibiting BP uptake by various organs including the kidney. Despite the substantial suppression, BP exclusion within 270 min after 6RBH₄ administration was almost the same in “BH₄ alone” and “BH₄ + Pbc” rats. This was explained by the fact that the concomitantly high concentration of plasma BP caused by the Pbc treatment speeded up the glomerular filtration. It should be noted that the tubular secretion was enhanced when mediated by a drug-sensitive transporter(s) presumably with large capacity but low affinity. This infers that the putative transporter only functions when the plasma BP concentration is high. Actually, it was demonstrated that the dominancy of epithelial secretion over glomerular filtration occurred only when the plasma BP concentration was 10 times higher than ordinary levels [12, 13]. In this context, adjusting the formula or recipe for 6RBH₄ administration so as to avoid the tubular secretion by maintaining the plasma BP level around the critical concentration (~ 10-fold) may result in a much higher efficiency in bodily replacement of BH₄.

We previously described excretion of BP in the bile as a route for its entero-hepatic circulation [14]. In the present experiment following experimental procedure (A) in the Materials and

Table 2. The BH₄% in the blood, liver and kidney after 6RBH₄ administration.

| Time | Rat group | Blood | Liver | Kidney |
|------|-----------|-------|-------|--------|
|      |           | %     |       | %      | %      | %      | %      | %      | %      | %      | %      |
| 30 min | BH₄ alone | 93.9 ±0.6 | 98.7 ±0.1 | ** | 98.0 ±0.2 | **, n.s. | |
|       | BH₄ + Pbc | 89.2 ±0.7 | 98.7±0.1 | ** | 97.7 ±0.3 | **, n.s. | |
|       | BH₄ + CsA | 94.4 ±0.1 | 98.6 ±0.1 | ** | 98.1 ±0.4 | **, n.s. | |
| 120 min | BH₄ alone | 96.2 ±0.1 | 98.8 ±0.1 | ** | 98.6 ±0.3 | **, n.s. | |
|        | BH₄ + Pbc | 87.6 ±3.7 | 98.1 ±0.1 | n.s. | 89.2 ±5.5 | n.s., n.s. | | 89.2 ±5.5 | n.s., n.s. |
|        | BH₄ + CsA | 95.3 ±0.1 | 98.5 ±0.1 | ** | 98.4 ±0.1 | **, n.s. | |

The BH₄% in organs 30 min and 120 min after administration of 6RBH₄ with or without prior treatment with Pbc or CsA. For each rat group, differences in the organ BH₄% between blood, liver and kidney were analyzed by Tukey’s multiple testing method. BH₄% values are means ± S.E. and apply to Fig 5.

1) Difference between the BH₄% of time-matched blood vs. liver or kidney. ** P<0.01 significance, suggesting the BH₄% is higher than that of blood; “n.s.”, not significant.

2) Difference between the BH₄% of time-matched liver vs. kidney.

doi:10.1371/journal.pone.0164305.t002
Methods

In the Methods section, we removed all the bile and did not let it return to the liver. After 6RBH₄ administration, the bile BP increased along with the liver BP levels in the presence or absence of Pbc. Further, the BH₄% of the bile BP was persistently high, similar to the high BH₄% in the liver, either in the presence or absence of Pbc. Pbc might have inhibited BP uptake by the liver cells but might not have affected its release into the bile. We also noted that the cumulative excretion of BP in the bile was only about 3% that in the urine. Although it was reported that biliary excretion was a major route for the elimination of various drugs or their metabolites [24, 25], our findings revealed that it was rather a minor route with regard to the bodily loss of administered BH₄. However, the fact that the liver excels in secreting BP with a high BH₄% suggests that it has some role in BH₄ secretion into the gut; however, the physiological function of the bile BH₄ remains elusive.

Distribution and retention of administered 6RBH₄ in the organs

Hoshiga et al. [26] described the body-wide distribution of exogenous BH₄ which they detected by means of whole body autoradiography after i.v. injection of a tracer amount of (6R)-[U-¹⁴C]BH₄. They found the densest deposition of radioactivity to be in the kidney at 2 hours. Among large organs, the liver had the next densest deposition, while deposition in the brain and striated muscles was unremarkable. Hayashi et al. (in a Japanese journal [27]) described BP distribution after administration of 6RBH₄. According to their report, the BP contents of various organs including the liver were all highest at 30 min (the earliest sampling) and the BP amount in each organ decreased gradually over the course of the 24-hour experiment, suggesting that the uptake phase of BP, in terms of the net balance of uptake minus release, had been virtually completed in organs within the first 30 min. In our present research, we treated rats with Pbc or CsA prior to administration of 6RBH₄ with the purpose of examining the effect of these inhibitors on BH₄ uptake by various organs and the respective differences in the BH₄%. We started sampling at 30 min, assuming that the net uptake had already been completed by the time that BP was circulating throughout the body based on the data reported by Hayashi et al. above. The retention of the BP accumulated in the liver was longer than that of the kidney and brain, and that of RBCs in the blood. Taking its large mass into account, the liver seemed to function as a large reservoir of the administered BH₄ for gradual supply to peripheral tissues including the vasculature. This meant that, over the long period, the liver released BP gradually to the bile and presumably to the blood circulation.

Homeostasis of BH₂/BH₄ after administration of 6RBH₄

We observed that the BH₄% in the blood and urine was considerably decreased while the amount of tissue BP was prominently increased in the liver and kidney after 6RBH₄ administration. Further, Pbc, an uptake inhibitor, enhanced the BH₄% decrease in the blood and urine. These results raised two questions: first, why did the BH₄% decrease after 6RBH₄ administration and secondly, why did the uptake inhibitor promote a further decrease.

As to the first question, we consider that the answer lies in the mechanisms by which cells take up extracellular BH₄. BH₄ is barely able to cross the cell membrane in its tetrahydro-form because as an active coenzyme, it must remain inside the cell. Generally, the cell membrane works as a strong barrier against the in and out movement of such a hydrophilically active coenzyme as BH₄. For the uptake of extracellular BH₄, a complex series of events must occur [28]. First, BH₄ is oxidized, the resultant BH₂ is taken up by the cell, an action presumably mediated by transporters, and then BH₂ is reduced back to BH₄ through the salvage pathway. Although the location and manner of the rapid oxidation of the administered 6RBH₄ was ambiguous, the systemic oxidation of BH₄ to BH₂ in vivo was clearly demonstrated by the
appearance of a BH$_2$ surge in the blood immediately after 6RBH$_4$ administration in the mouse [19]. In the present study using rats under anesthesia, we did not observe such a prominent BH$_2$ surge after 6RBH$_4$ administration, but noted instead a significant decrease in the BH$_4$%, in other words, an increase in BH$_2$ relative to BH$_4$, in the blood and urine. The moderate but long-lasting decrease in the BH$_4$% in rats seemed to result from essentially the same process responsible for the rapid appearance of the BH$_2$ surge in mice. We consider that in both rats and mice, these outcomes reflect an inevitable compensatory response of the animal body toward exogenous BH$_4$.  

As for the second question, the answer is contained in the response to the first. The reducing action of BH$_2$ is an intracellular event but the BH$_2$ has to be brought into the cell from the plasma. Therefore, BH$_2$ uptake by the tissues is a prerequisite for scavenging blood BH$_2$. Although the uptake transporters have not all been identified, at least we know that two equilibrative nucleoside transporters called ENT1 (SLC29A1) and ENT2 (SLC29A2) are able to mediate BH$_2$ relocation favoring the other dihydropterin-compound, sepiapterin, but not BH$_4$ [29]. Some portion of the BP uptake observed in this study was obviously mediated by another transporter(s) because ENT1 and ENT2 are sensitive to nitrobenzylthioinosine (NBMPR) but not to Pbc to our knowledge. The fact that the blockade of BP uptake by Pbc enhanced a further decrease in the blood BH$_4$% suggests that BH$_2$ was effectively or even preferentially taken up by the putative transporter(s) which was sensitive to Pbc. Since the salvage pathway is thermodynamically favored to proceed, the internalized BH$_2$ is readily scavenged as in the push-pull accumulation of BH$_4$ [18, 30]. As for extracellular BH$_4$, however, we have not uncovered any form of active transport that would allow it to directly accumulate in the cell against a concentration gradient in a tetrahydro-form. A passive transport could not contribute to BH$_4$ accumulation against the concentration gradient even if a transporter(s) could potentially move it across the plasma membrane.

The endogenous content and the capacity to uptake BH$_4$ was prominent in the liver and kidney (Table 1). This suggested to us that most BH$_2$ scavenging might have occurred in these organs. It was noted that the BH$_4$% in these organs did not decrease when the BH$_4$% was lowered in the blood and urine after 6RBH$_4$ administration. BP uptake by the liver was considerably inhibited by prior treatment with Pbc and it was accompanied by an even greater decline in the BH$_4$% of the blood and urine. This observation was explained by inhibition of the uptake process by the drug and the consequent inability of the liver to convert BH$_2$ to BH$_4$. Taking the liver's large capacity for BP uptake into account, this organ might have played the greatest role in removing the scavenged BH$_2$. Considering that the kidney excreted urine with a consistently higher BH$_4$% than that of the plasma, the strong ability of the kidney to scavenge BH$_2$ was also noted. The pronounced decrease in the BH$_4$% in the urine in the presence of Pbc suggested reduced BH$_2$ scavenging in the kidney. This Pbc sensitivity indicates that the BH$_2$ scavenging occurred during the transit across the tubular epithelium for secretion. In this manner, the kidney was able to scavenge BH$_2$ from the plasma. Additionally, the kidney might have released a large amount of BH$_2$-scavenged "clean" BP back to the plasma resulting in a rise in the plasma BH$_4$%, but our experiment was not designed to show this explicitly. The details of tubular secretion with regard to the relevant transporter for BH$_2$ scavenging remain elusive.

When our focus was limited to classic enzyme pathways, the salvage pathway of BH$_2$ was seen as a mechanism for maintaining BH$_4$ levels against a loss of BH$_4$ cofactor activity. The salvage pathway, coupled with uptake of the extracellular precursor, should now be considered as an innate mechanism for scavenging BH$_2$, a mechanism which covers a wide range of interactions between cells and their environment throughout the body. The liver and kidney are the
major sites of BH$_2$ scavenging under conditions of 6RBH$_4$ administration owing to their large-scale uptake of blood BP.

Remarks concerning NOS dysfunction

In clinical practice, 6RBH$_4$ has been prescribed to patients with an inherited BH$_4$ deficiency as well as to many with BH$_4$-responsive phenylketonuria. On a search of the literature, we did not encounter any mention of a serious adverse effect of long-term administration in these patients [31]. In another area of medicine, BH$_4$ supplementation has also drawn increased attention with respect to whether it ameliorates NOS dysfunction in the cardiovascular system as a high BH$_2$/BH$_4$ ratio, representing a type of oxidative stress, is a great risk factor. Studies in this area are concerned with the homeostasis of the BH$_2$/BH$_4$ ratio, an inverse expression of the BH$_4$%.

The administration of 6RBH$_4$ is followed by a lowering of the blood BH$_4$% as an inevitable compensatory reaction, as discussed above. In the case of the lowering of the BH$_4$% in the blood, endothelial cells are the ultimate interface in all organs and are active sites of nitric oxide (NO) production regulating vascular contraction. In fact, we previously demonstrated that ENT1 and ENT2 were able to mediate cell membrane permeation of sepiapterin preferentially, permeation of BH$_2$ moderately and that of BH$_4$ only slightly [29]. Furthermore, we localized ENT2 on the vascular lumen side of endothelial cells [32], suggesting that these cells are ready to uptake increased amounts of BH$_2$. Owing to two characteristic properties of the enzyme NOS, once in the cell, BH$_2$ must be converted to BH$_4$ promptly. 1) The affinity of NOS for BH$_2$ is not particularly low compared to its affinity for BH$_4$; in the case of an nNOS, an isoform of eNOS, the affinity to BH$_2$ and to 6RBH$_4$ was as close as 1:10 [33]. 2) BH$_2$-bound NOS is unable to maintain an active dimer and uncouples O$_2$ reduction to produce superoxide [34–36]. In this context, dihydrofolate reductase is the key enzyme for maintaining a BH$_2$/BH$_4$ ratio sufficiently low for sound NO production. However, endothelial cell-derived culture cells, especially of human origin, are poorly furnished with dihydrofolate reductase [37–39], suggesting that the original endothelial cells in the human vasculature have the same vulnerability. Consequently, a high BH$_2$/BH$_4$ ratio in the plasma causes eNOS to uncouple locally in endothelial cells and leads to a negative spiral favoring BH$_4$ oxidation [40]. In this study, we noticed that after 6RBH$_4$ administration, the blood BH$_4$% persistently dropped below 90%, that is, the BH$_2$ to BH$_4$ ratio became less than 1:10 even in the “BH$_4$ alone” group, while it was maintained at ca. 95% in untreated rats. We also noted that Pbc, a widely prescribed anti-gout drug, further increased the blood BH$_2$ when used with 6RBH$_4$. It is interesting that many experimental studies support the use of 6RBH$_4$ administration as a means of ameliorating cardiovascular symptoms, however, translation of these studies to human patients remains limited [41]. The stated vulnerability of endothelial cells suffering from a lowering of the blood BH$_4$% might represent a great risk factor for possible exacerbation of cardiovascular dysfunction in cases in which 6RBH$_4$ is regularly administered repeatedly at a dose of 5–20 mg/kg/day.

We conclude that supplementation with BH$_4$ by means of administration of 6RBH$_4$ has remarkably low efficacy due to its rapid loss through the urine, nonetheless it is still effective in raising BH$_4$ levels in peripheral organs. However, considering the general vulnerability of the cardiovascular system to an increasing BH$_2$/BH$_4$ ratio, regular administration of 6RBH$_4$ may spoil the desired effect of countering NOS dysfunction by lowering the BH$_4$%. The compensatory lowering of the BH$_4$% must be avoided in future improvements to the therapeutic composition. Take together improving 6RBH$_4$ preparations to allow for sustained distribution while avoiding a steep elevation in plasma might help to realize the potential benefit of BH$_4$ supplementation.
Acknowledgments

We wish to thank R Fujii, N Nishimoto, Y Suetsaka, Y Aoshima, M Fukasawa, M Ishikawa, M Nito, T Okamoto, E Saito, K Sekihata, T Sumi, Y Taiga, R Tanaka, A Watanabe, and S Yamanzaki for their efforts and collaboration in the Student Research Program 2007–2009, Department of Biosciences, Teikyo University of Science and Technology. We are grateful to Dr William Campbell and Catherine Campbell for editing our draft for correct English usage.

Author Contributions

Conceptualization: AO HH.
Data curation: AO HH.
Formal analysis: AO HH TH.
Funding acquisition: HH SA TT AO.
Investigation: YS AO HH.
Methodology: AO YS HH.
Project administration: HH AO.
Resources: HH YS AO.
Supervision: HH.
Validation: AO HH.
Visualization: AO HH.
Writing – original draft: AO HH.
Writing – review & editing: AO HH SA TH MN TT.

References

1. Kaufman S. The Structure of the phenylalanine-pyridoxylation cofactor. Proc Natl Acad Sci U S A. 1963 Dec; 50:1085–93. doi: 10.1073/pnas.50.6.1085 PMID: 14096182.
2. Nagatsu T, Levitt M, Udenfriend S. Tyrosine hydroxylase: the initial step in norepinephrine biosynthesis. J Biol Chem. 1964 Sep; 239:2910–7. PMID: 14216443.
3. Lovenberg W, Jequier E, Sjoerdsma A. Tryptophan hydroxylation: measurement in pineal gland, brain-stem, and carcinoid tumor. Science. 1967 Jan 13; 155(759):217–9. doi: 10.1126/science.155.3759.217 PMID: 6015530. Epub 1967/01/13. eng.
4. Ichiyama A, Nakamura S, Nishizuka Y, Hayashi O. Enzyme studies on the biosynthesis of serotonin in mammalian brain. J Biol Chem. 1970 Apr 10; 245(7):1699–709. PMID: 5309585. Epub 1970/04/10. eng.
5. Blau N. Sapropterin dihydrochloride for the treatment of hyperphenylalaninemia. Expert Opinion on Drug Metabolism & Toxicology. 2013; 9(9):1207–18. doi: 10.1517/17425255.2013.804064 PMID: 23705856.
6. Bartholome K, Byrd DJ, Kaufman S, Milstien S. Atypical phenylketonuria with normal phenylalanine hydroxylase and dihydropteridine reductase activity in vitro. Pediatrics. 1977 May; 59(5):757–61. PMID: 300866. Epub 1977/05/01. eng.
7. Schaub J, Daumling S, Curtius HC, Niederwieser A, Bartholome K, Viscontini M, et al. Tetrahydrobiopterin therapy of atypical phenylketonuria due to defective dihydrobiopterin biosynthesis. Arch Dis Child. 1978 Aug; 53(8):674–6. doi: 10.1136/adc.53.8.674 PMID: 708106. Pubmed Central PMCID: 1545051. Epub 1978/08/01. eng.
8. Kure S, Hou DC, Ohura T, Iwamoto H, Suzuki S, Sugiyama N, et al. Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. J Pediatr. 1999 Sep; 135(3):375–8. doi: 10.1016/S0022-3476(99)7038-1 PMID: 10484807. Epub 1999/09/15.
13. Ohashi A, Suetake Y, Saeki Y, Harada T, Aizawa S, Hasegawa H. Corrigendum to “Rapid clearance of supplemented tetrahydrobiopterin is driven by high-capacity transporters in the kidney” [Mol Genet Metab. 2013; 108:107. doi: 10.1016/j.ymgme.2012.11.007 PMID: 22318121]

14. Heintz C, Cotton RG, Blau N. Tetrahydrobiopterin, its mode of action on phenylalanine hydroxylase, and importance of genotypes for pharmacological therapy of phenylketonuria. Human Mutation. 2013 Jul; 34(7):927–36. PMID: 23559577. Epub 2013/04/06. eng. doi: 10.1002/humu.22320

15. Wettstein S, Underhaug J, Perez B, Marsden BD, Yue WW, Martinez A, et al. Linking genotypes database with locus-specific database and genotype-phenotype correlation in phenylketonuria. European Journal of Human Genetics: EJHG. 2014 Jun 18. doi: 10.1038/ejhg.2014.114 PMID: 24939588.

16. Ohashi A, Sugawara Y, Mamada K, Harada Y, Sumi T, Anzai N, et al. Membrane transport of sepiapterin and dihydrobiopterin by equilibrative nucleoside transporters: a plausible gateway for the salvage pathway of tetrahydrobiopterin biosynthesis. Mol Genet Metab. 2011; 102:18–28. doi: 10.1016/j.ymgme.2010.09.005 PMID: 20956085

17. Sawabe K, Saeki Y, Ohashi A, Mamada K, Wakasugi KO, Matsuoka H, et al. Tetrahydrobiopterin in intestinal lumen: Its absorption and secretion in the small intestine and the elimination in the large intestine. J Inherit Metab Dis. 2009 Feb; 32(1):79–85. PMID: 19031009. Epub 2008/11/26. Eng. doi: 10.1007/s10545-008-0964-0

18. Hsu V, de LTVM, Zhao P, Zhang L, Zheng JH, Nordmark A, et al. Towards quantitation of the effects of renal impairment and probenecid inhibition on kidney uptake and efflux transporters, using physiologically based pharmacokinetic modelling and simulations. Clinical Pharmacokinetics. 2014 Mar; 53 (3):283–93. doi: 10.1007/s40262-013-0117-y PMID: 24214317. Pubmed Central PMCID: PMC3927066. Epub 2013/11/12. eng.

19. Inoue F, Hasegawa H, Nishimura M, Yanagisawa M, Ichiyama A. Distribution of 5-hydroxytryptamine (5HT) in tissue of a mutant mouse deficient in mast cell (W/Wv). Demonstration of the contribution of mast cells to the 5HT content in various organs. Agents Actions. 1985 Jul; 16(5):295–301. doi: 10.1007/bf01982861 PMID: 4050611. Epub 1985/07/01. eng.

20. Fukushima T, Nixon JC. Analysis of reduced forms of biopterin in biological tissues and fluids. Anal Biochem. 1980 Feb; 102(1):176–88. doi: 10.1016/0003-2697(80)90336-x PMID: 7356152. Epub 1980/02/01. eng.

21. Ohashi A, Sugawara Y, Mamada K, Harada Y, Sumi T, Anzai N, et al. Membrane transport of sepiapterin and dihydrobiopterin by equilibrative nucleoside transporters: a plausible gateway for the salvage pathway of tetrahydrobiopterin biosynthesis. Mol Genet Metab. 2011; 102:18–28. doi: 10.1016/j.ymgme.2010.09.005 PMID: 20956085

22. Sawabe K, Wakasugi KO, Hasegawa H. Tetrahydrobiopterin uptake in supplemental administration: elevation of tissue tetrahydrobiopterin in mice following uptake of the exogenously oxidized product 7,8-dihydrobiopterin and subsequent reduction by an anti-folate-sensitive process. J Pharmacol Sci. 2004 Epub 2004 Oct 2.; 96(2):124–33. doi: 10.1254/jphs.fp0040280 PMID: 15467264.

23. Brand MP, Hyland K, Engle T, Smith I, Heales SJ. Neurochemical effects following peripheral administration of tetrahydropterin derivatives to the hph-1 mouse. J Neurochem. 1996 Mar; 66(3):1150–6. doi: 10.1046/j.1471-4159.1996.66031150.x PMID: 8769878.

24. Levine RA, Zoephel GP, Niederwieser A, Curtius HC. Entrance of tetrahydropterin derivatives in brain after peripheral administration: effect on biogenic amine metabolism. J Pharmacol Exp Ther. 1987 Aug; 242(2):514–22. PMID: 3612548. Epub 1987/08/01. eng.

25. Miwa S, Watanabe Y, Hayashi O. 6R-L-erythro-5,6,7,8-tetrahydrobiopterin as a regulator of dopamine and serotonin biosynthesis in the rat brain. Arch Biochem Biophys. 1985 May 15; 239(1):234–41. doi: 10.1016/0003-2697(85)90083-1 PMID: 4004257. Epub 1985/05/15. eng.

26. Koshimura K, Murakami Y, Tanaka J, Kato Y. The role of 6R-tetrahydrobiopterin in the nervous system. Prog Neurobiol. 2000 Jul; 61(4):415–38. doi: 10.1016/s0301-0082(99)00059-3 PMID: 10727782. Epub 2000/03/23. eng.

27. Singhvi SM, Pan HY, Morrison RA, Willard DA. Disposition of pravastatin sodium, a tissue-selective HMG-CoA reductase inhibitor, in healthy subjects. Br J Clin Pharmacol. 1990 Feb; 29(2):239–43. doi: 10.1111/j.1365-2125.1990.tb03626.x PMID: 2106337. Pubmed Central PMCID: 1380090.

28. Hagenbuch B, Gui C. Xenobiotic transporters of the human organic anion transporting polypeptide (OATP) family. Xenobiotica. 2008 Jul; 38(7–8):778–801. PMID: 1866430. doi: 10.1080/00498250801966951
26. Hoshiga M, Hatakeyama K, Watanabe M, Shimada M, Kagamiyama H. Autoradiographic distribution of [14C]tetrahydrobiopterin and its developmental change in mice. J Pharmacol Exp Ther. 1993; 267(2):971–8. PMID: 8246172

27. Hayashi T, Ogata A, Takehisa M, Komoriya K, Ohnuma N. Studies on metabolism and disposition of sapropterin hydrochloride (SUN 0588) L-erythro-tetrahydrobiopterin hydrochloride in rats. Kiso to Rinsho. 1992; 26(8):3471–95.

28. Hasegawa H, Sawabe K, Nakanishi N, Wakasugi OK. Delivery of exogenous tetrahydrobiopterin (BH4) to cells of target organs: role of salvage pathway and uptake of its precursor in effective elevation of tissue BH4. Mol Genet Metab. 2005 Dec; 86 Suppl 1:S2–10. doi: 10.1016/j.ymgme.2005.09.002 PMID: 16256391.

29. Ohashi A, Fukumuro M, Sawabe K, Mamada K, Sugawara Y, Matsuoka H, et al. Transcellular relocation of tetrahydrobiopterin across Caco-2 cells: A model study of tetrahydrobiopterin absorption through epithelial cells of intestinal mucosa. J Inherit Metab Dis. 2009 Nov 3Feb; 32(1):73–8. PMID: 19793179. Epub 2009/05/13. doi: 10.1007/s10545-008-0961-3

30. Blau N, Longo N. Alternative therapies to address the unmet medical needs of patients with phenylketonuria. Expert Opin Pharmacother. 2015 Apr; 16(6):791–800. PMID: 25660215. Epub 2015/02/11. eng. doi: 10.1517/14656566.2015.1013030

31. Stuehr D, Pou S, Rosen GM. Oxygen reduction by nitric-oxide synthases. J Biol Chem. 2001 May 4; 276(18):14533–6. doi: 10.1074/jbc.r100011200 PMID: 11279231. Epub 2001/03/30. eng.

32. Schmidt K, Kolesnik B, Gorren AC, Werner ER, Mayer B. Cell type-specific recycling of tetrahydrobiopterin by dihydrofolate reductase explains differential effects of 7,8-dihydrobiopterin on endothelial nitric oxide synthase uncoupling. Biochem Pharmacol. 2014 Aug 1; 90(3):246–53. PMID: 24883258. Pubmed Central PMCID: 4099517. doi: 10.1016/j.bcp.2014.05.010

33. Crabtree MJ, Hale AB, Channom KM. Dihydrofolate reductase protects endothelial nitric oxide synthase from uncoupling in tetrahydrobiopterin deficiency. Free Radic Biol Med. 2011 Oct; 53(4):559–63. PMID: 21458460. Epub 2011/04/05. eng. doi: 10.1016/j.freeradbiomed.2011.03.010

34. Moens AL, Kietadisorn R, Lin JY, Kass D. Targeting endothelial and myocardial dysfunction with tetrahydrobiopterin. J Mol Cell Cardiol. 2011 Oct; 51(4):559–63. PMID: 21458460. Epub 2011/04/05. eng. doi: 10.1016/j.yjmcc.2011.03.009