Measurement of Tetrahydrobiopterin in Animal Tissue Samples by HPLC with Electrochemical Detection—Protocol Optimization and Pitfalls

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Abstract: Tetrahydrobiopterin (BH4) is an essential cofactor of all nitric oxide synthase isoforms, thus determination of BH4 levels can provide important mechanistic insight into diseases. We established a protocol for high-performance liquid chromatography/electrochemical detection (HPLC/ECD)-based determination of BH4 in tissue samples. We first determined the optimal storage and work-up conditions for authentic BH4 and its oxidation product dihydrobiopterin (BH2) under various conditions (pH, temperature, presence of antioxidants, metal chelators, and storage time). We then applied optimized protocols for detection of BH4 in tissues of septic (induced by lipopolysaccharide [LPS]) rats. BH4 standards in HCl are stabilized by addition of 1,4-dithioerythritol (DTE) and diethylenetriaminepentaacetic acid (DTPA), while HCl was sufficient for BH2 standard stabilization. Overnight storage of BH4 standard solutions at room temperature in HCl without antioxidants caused complete loss of BH4 and the formation of BH2. We further optimized the protocol to separate ascorbate and the BH4 tissue sample and found a significant increase in BH4 in the heart and kidney as well as higher BH4 levels by trend in the brain of septic rats compared to control rats. These findings correspond to reports on augmented nitric oxide and BH4 levels in both animals and patients with septic shock.

Keywords: tetrahydrobiopterin; HPLC with electrochemical detection; oxidative stress; sepsis

1. Introduction

Tetrahydrobiopterin (BH4, sapropterin) is a cofactor of the three aromatic amino acid hydroxylase enzymes and nitric oxide synthases (NOS). The hydroxylase enzymes convert aromatic amino acids such as phenylalanine, tyrosine, and tryptophan to precursors of dopamine and serotonin, which act as major monoamine neurotransmitters [1]. The NOS enzyme family produces nitric oxide (NO), a mediator of endothelium-dependent vasodilation, inhibitor of platelet aggregation and regulator of smooth muscle tone, cell growth and differentiation [2]. BH4 is sold under the brand names Kuvan and Bioten for supplementation in BH4 deficiency caused by genetic inactivity of GTP-cyclohydrolase-I (GTPCH-I) or 6-pyruvoyltetrahydropterin synthase (PTPS), both enzymes involved in BH4 synthesis as encountered in phenylketonuria [3].
BH4 is an important regulator of cardiovascular development and homeostasis [4]. BH4 deficiency has been reported in almost all cardiovascular diseases and has been suggested to play an important role in the development of endothelial dysfunction and progression of atherosclerosis [4–8]. Oxidative loss of BH4 triggers the uncoupling of endothelial NOS (eNOS) in the conditions of oxidative stress that are present in hypertension, diabetes, hypercholesterolemia and atherosclerosis [2,9–12]. Oxidation of BH4, e.g., in the presence of peroxynitrite, leads to formation of 7,8-dihydrobiopterin (BH2) [13,14], which can be further oxidized to bioppterin and other products. Since BH4 bioavailability is tightly linked to regulation of eNOS activity, the levels of BH4 have a strong impact on vascular function, cardiovascular health and the adhesion/infiltration of immune cells [15–18]. On the other hand, induction of BH4 synthesis is a hallmark of severe inflammatory conditions, as observed in the setting of endotoxemia (e.g., by lipopolysaccharide [LPS]) and septic shock [19–21]. A similar increase in BH4 synthesis and nitric oxide formation was observed in a more moderate model of cardiovascular inflammation, mice with angiotensin-II-induced arterial hypertension [18]. Generally, inflammation is associated with upregulation of GTPCH-I, providing higher BH4 concentrations for inducible nitric oxide synthase (iNOS) [18,22].

In light of the aforementioned central role of BH4 in endothelial dysfunction as well as inflammation-associated complications, the exact determination of BH4 is of great importance. BH4 can be detected by indirect methods upon the differential oxidation of BH4 and BH2 to fluorescent bioppterin (iodine method) as well as by direct methods using post-column electrochemical oxidation or MS/MS-based detection. Indirect methods rely on liquid chromatography coupled to UV/Vis or fluorescence detection of BH4 oxidation products [23]. Here, we used the HPLC/ECD method for the reliable (and direct) detection of BH4 and BH2 concentrations in standards as well as BH4 levels in samples of control and septic rats. We also used the HPLC/ECD method for evaluation of the optimal storage and work-up conditions for BH4- and BH2-containing samples.

2. Materials and Methods

2.1. Chemicals

Tetrahydro-L-biopterin (BH4) hydrochloride and 7,8-dihydro-L-biopterin (BH2) hydrochloride were obtained from Cayman Chemical Company (Ann Arbor, MI, USA) (CAS: 69056-38-8). Hydrogen peroxide 30% solution (CAS: 7722-84-1), 1,4-dithioerythritol ≥ 99.0% (DTE) (CAS: 6892-68-8), diethylenetriaminepentaacetic acid 98% (DTPA) (CAS: 67-43-6), LPS from Escherichia coli (L-4005) and Salmonella typhosa (L6386), potassium dihydrogen phosphate (# P018.2) and phosphorus acid (# 9079.1) were obtained from Sigma, Merck KGaA, Darmstadt, Germany.

2.2. Stability of BH4 and BH2 Standard Stock Solutions under Different Storage Conditions

BH4 and BH2 standard stock solutions of 1 mM were prepared in 100 µM HCl with or without 1 mM DTE and 1 mM DTPA, which were then divided and further diluted to 100 µM for overnight storage and up to 2 weeks at different temperatures (4 °C, −20 °C and −80 °C). Some standard stock solutions were incubated with 1 mM hydrogen peroxide overnight at 4 °C in order to mimic the autoxidation of BH4 at ambient oxygen concentrations. After applying the different storage conditions and times, the samples were subjected to HPLC/ECD analysis.

2.3. Animals

All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health and approval was granted by the Ethics Committee of the University Hospital Mainz and the Landesuntersuchungsamt Rheinland-Pfalz (Koblenz, Germany; permit number: 23 177-07/G 18-1-001). Male Wistar rats (6 weeks old, 300 g, Charles River Laboratories, Sulzfeld, Germany) were used for the study and all efforts were made to minimize suffering. Diabetes was induced
with a single injection of streptozotocin (60 mg/kg) in the dorsal vein of the penis with incubation of animals for 6 weeks [24,25], while sepsis was induced by intraperitoneal injection of LPS (from Escherichia coli and Salmonella typhimurium in ratio 3:1, 10 mg/kg) 12 h before sacrifice [26,27]. Animals were killed under isoflurane anesthesia by transection of the diaphragm and exsanguination. Aorta, heart, brain, liver and kidney tissues were harvested for further analysis.

2.4. HPLC/ECD

For the electrochemical detection of BH$_4$ and BH$_2$, an UltiMate 3000 system with Dionex™ CoulArray™ (Coulometric Array Detector) (Thermo Fisher Scientific GmbH, Dreieich, Germany) was used—a high-quality instrument designed for high sensitivity detection of electroactive molecules. The system is controlled by two different software programs: Chromeleon Chromatography Management System (Chromeleon) software and CoulArray software. Chromeleon software controls the injection volume of sample, flow, mobile phase gradient and functions of the autosampler. The CoulArray software controls the temperature of the column and the two coulometric multi-channel cells, as well as the setup of different electrochemical potentials. A coulometric cell with a large surface area consisting of porous graphite electrode material allows complete oxidation (or reduction) of the electrochemically active molecules, minimizing the noise and providing enhanced sensitivity. Each sample run ends with a short cleaning procedure of the electrochemical cells (setting all cells to 800 mV).

For detection of BH$_4$ (protocol A), potentials of 0, +150, +280, +365, +600 mV (vs. palladium reference electrode) were used. The BH$_4$ peak yielded the most pronounced signal at 280 mV, whereas the BH$_2$ peak showed the highest signal at 600 mV. For separation, an analytical column Synergi Polar-RP (Phenomenex, Torrance, CA, USA, 4 µm, 80 Å, 4.6 × 250.0 mm) was used at 37 °C column temperature (4 °C autosampler rack temperature). A sample volume of 20 µL was injected. The mobile phase consisted of 50 mM potassium phosphate, pH 2.6, with 0.1 mM DTE and 0.1 mM DTPA using an isocratic protocol, with the flow rate set at 0.7 mL/min. External and internal BH$_4$/BH$_2$ standards (by “spiking”) were used for peak identification and quantification. Typical retention times were 5.3 min for BH$_4$ and 9.2 min for BH$_2$. Protocol A was adapted from previous work [28,29]. Unfortunately, ascorbate co-eluted with BH$_4$ in this HPLC method.

For the analyses of tissue samples, we established a second protocol, protocol B, with a clear separation of BH$_4$ and ascorbate peaks. 50 mM potassium phosphate was set to pH 4.5 (based on other reports using higher pH of the mobile phase [30,31]), without DTE and DTPA using isocratic protocol with flow rate set at 1 mL/min while cell potentials were set to 0 and +150 mV. This condition provided better separation of interfering peaks in the animal tissue with BH$_4$ eluting at 4.6 min with the most pronounced signal observed at 0 mV. All experimental parameters for optimized HPLC/ECD-based detection of BH$_4$ and BH$_2$ used in both protocols are shown in Table 1.

2.5. Animal Tissue Preparation

After sacrifice, rat tissues (aorta, heart, brain, kidney and liver) were immediately washed and briefly stored in ice-cold homogenization solution consisting of 50 mM potassium phosphate buffer pH 2.6 with 1 mM DTE and 1 mM DTPA. Tissue was cleaned from extra fat (aorta was cleaned in homogenization buffer under the microscope on ice). The wet weight of all the tissues was measured before homogenization. Roughly, half of a heart (~700 mg), half of a kidney (~1000 mg), similar piece of a liver or a brain (~1000 mg) and the whole aorta (~150 mg) were used for BH$_4$ analysis. Samples were cut into small pieces, followed by glass–glass homogenization at 4 °C upon addition of 1 mL of homogenization buffer for heart, brain, kidney and liver tissue and 700 µL for aorta. The samples were centrifuged for 15 min at 15,000× g. Supernatant (slightly colored) was taken and centrifuged again through 10 kDa cut-off filters for 45 min 20,000× g. After centrifugation the completely colorless supernatant was loaded to the autosampler. All centrifugation
steps and the autosampler were kept at 4 °C. Supernatant of the first centrifugation step was used for quantification of protein content by Lowry method. All work-up steps and conditions are summarized in Figure 1.

Table 1. Experimental parameters for HPLC/ECD-based detection of BH4 and BH2 used in this study.

| Parameters                              | Specification                                      |
|-----------------------------------------|----------------------------------------------------|
| HPLC system                             | Dionex UltraMate 3000 (Thermo Fisher Scientific)   |
| Column                                  | Synergi Polar, 250 mm × 4.6 mm, 4 µm, 80 Å (Phenomenex) |
| Column temperature                      | 37 °C                                              |
| Mobile phase                            | a 50 mM potassium phosphate, pH 2.6, 0.1 mM DTE, 0.1 mM DTPA  
                                           b 50 mM potassium phosphate, pH 4.5 |
| Flow rate                               | a 0.7 mL/min  
                                           b 1.0 mL/min |
| Run time per sample                     | 15 min                                             |
| Injection volume                        | 20 µL                                              |
| Autosampler temperature                 | 4 °C                                               |
| Detector                                | Dionex CoulArray™ (Thermo Fisher Scientific)       |
| Autosampler temperature                 | 4 °C                                               |
| Detectors                                |                                                    |
| Electrode potentials (mV vs. palladium reference electrode) | a 0; +150; +280; +365; +600  
                                           b 0; +150; +280 |
| BH4 quantification channel(s)           | a 150 mV + 280 mV  
                                           b 0 mV |
| BH2 quantification channel(s)           | a 600 mV  
                                           b 280 mV |
| Accuracy (deviation from the expected calibration curve value) | a 7.99% (0 mV, 1 µM BH4, n = 16)  
                                           b −3.11% (280 mV, 1 µM BH2, n = 15)  
                                           c 11.27% (0 mV, 1 µM BH2, n = 16)  
                                           d 3.36% (280 mV, 1 µM BH2, n = 15)  
                                           e 3.13 ± 0.08 nA (0 mV, BH4, n = 10)  
                                           f 0.49 ± 0.016 nA (280 mV, BH2, n = 10) |
| Precision (deviation from the measured mean) | a 4.9 nA, 0.38 pmol (0 mV, BH4)  
                                           b 9.4 nA, 1.35 pmol (0 mV, BH4)  
                                           c 1.5 nA, 0.11 pmol (280 mV, BH2) |
| Noise                                   | b                                                   |
| Limit of quantification, peak height and quantity of material (S/N = 10) | b                                                   |
| Limit of detection, peak height and quantity of material (S/N = 3) | b                                                   |

a Protocol A; b Protocol B (optimized for separation of BH4 and ascorbate in biological samples).

Figure 1. Scheme with work-up conditions for harvesting and processing of animal tissues. Created with Biorender.com (last accessed 14 June 2022).
2.6. Statistical Analysis

Results are expressed as mean \( \pm \) SD. Unpaired t-test was used for all BH\(_4\) data obtained in tissues; all showed normal distribution, but some failed equal variance where Welch correction was used. Non-parametric test was used for all BH\(_4\) data obtained in standard stock solutions (GraphPad Prism 9.0.1 for Windows.). \( p \)-Values < 0.05 were considered statistically significant.

3. Results

3.1. Standard Curve

BH\(_4\) was detected at the lower potentials of 150 mV and 280 mV and showed a peak at a retention time of 5.35 min, whereas BH\(_2\) was detected at the high potential of 600 mV and showed a peak at a retention time of 9.38 min (Figure 2A). Standard curves for both BH\(_4\) and BH\(_2\) were linear in the concentration range of 25 to 150 or 100 \( \mu \text{M} \) with \( R^2 \) values of >0.98 (Figure 2B).

![Figure 2. (A) Chromatograms of 100 \( \mu \text{M} \) BH\(_4\) and BH\(_2\) standards represented at oxidation potentials of 150, 280 and 600 mV for BH\(_4\) (retention time 5.4 min) and at the oxidation potential of 600 mV for BH\(_2\) (retention time 9.4 min). (B) Standard curves for BH\(_4\) and BH\(_2\) solutions, representing a linear range (25–150 and 25–100 \( \mu \text{M} \) concentration) with a linear regression correlation coefficient \( R^2 \) of >0.98 for both standards. Data are mean \( \pm \) SD of \( n = 4 \) independent measurements.](image-url)

3.2. Stability of Standards during Storage

Standards were prepared in 100 \( \mu \text{M} \) HCl (plus acidity from BH\(_4\) and BH\(_2\) hydrochlorides used) with or without 1 mM DTE and 1 mM DTPA. These standards were either measured directly after preparation or upon storage at room temperature, 4 \( ^\circ \text{C} \) or \(-80 \ ^\circ \text{C}\).
overnight, for 1 week or for 2 weeks. BH$_4$ was completely decomposed and BH$_2$ was formed upon overnight storage at room temperature when only dissolved in HCl solution. Even when stored at 4 °C, BH$_4$ showed appreciable degradation after one or 2 weeks of storage when only dissolved in HCl. The presence of DTE and DTPA stabilized BH$_4$ standards at all temperatures. Only at longer storage times (weeks) there was up to 20% unspecific loss of BH$_4$, even at −80 °C. BH$_2$ standards were stable under all conditions in HCl solution. Graphs for BH$_4$ and BH$_2$ signal response after storage under different conditions are shown in Figure 3.

Figure 3. (A) Detected BH$_4$ peak areas in solutions of BH$_4$ (100 µM) standards (quantified as the sum of 150 mV and 280 mV oxidation potentials, eluted at 5.4 min) upon storage under indicated conditions and time. (B) Peak areas of BH$_2$ detected (quantified at 600 mV oxidation potential, retention time 9.4 min) in the same samples. (C) Detected BH$_2$ peak areas in solutions of BH$_2$ (100 µM) standards (quantified at 600 mV oxidation potential, eluted at 9.4 min) upon storage under different conditions. Solvent conditions were either hydrochloric acid (HCl, 100 µM) or HCl (100 µM) with dithioerythritol (DTE, 1 mM) and diethylenetriaminepentaacetic acid (DTPA, 1 mM). Data are mean ± SD of the number of indicated independent measurements. Note: * means significantly different to fresh standard in HCl; § means significantly different to fresh standard in HCl with DTE/DTPA.
3.3. Oxidation of Standards

Oxidation of 100 µM BH₄ standard (prepared in 100 µM HCl solution) by 1 mM H₂O₂ was performed by overnight incubation at 4 °C. BH₄ was completely decomposed and there was notable formation of BH₂ as well as another, unidentified degradation product with the retention time of approximately 11 min (Figure 4).

**Figure 4.** (A) Representative chromatograms showing the changes in peak intensities due to BH₄, BH₂ and unidentified product (marked with “?” symbol, retention time approximately 11 min) in BH₄ (100 µM) solutions incubated in the presence or absence of H₂O₂ (1 mM). (B) Concentrations of BH₄ standard and formed BH₂ product (quantified as the sum of 150 mV and 280 mV oxidation potentials for BH₄, and only 600 mV for BH₂) were either measured in freshly prepared samples in 100 µM HCl or after incubation overnight at 4 °C or after incubation overnight at 4 °C in the presence of 1 mM H₂O₂ in 0.1 mM HCl. Data are mean ± SD of the number of indicated independent measurements. Note: * means significantly different to fresh BH₄; § means significantly different to overnight BH₄.
3.4. Animal Tissue

Using the first HPLC protocol A buffer and settings (50 mM potassium phosphate, pH 2.6, with 0.1 mM DTE and 0.1 mM DTPA), a signal was detected in all animal tissues (aorta, heart, kidney, liver and brain) that were eluted with the retention time of BH₄, which was only significantly reduced in aortic tissue of diabetic (STZ) rats (Figure 5A). BH₂ could not be detected in any of these tissues. We were, however, surprised by the magnitudes higher concentration of BH₄ in all of these tissues as compared to previously published data and realized that ascorbate often co-elutes with BH₄ and shows comparable electrochemical properties, as stressed previously [32]. Further analysis showed that, indeed, BH₄ and ascorbate standards co-eluted at the same time (Figure 5B). We therefore disregarded all data obtained from the tissues of the diabetic rats.

Figure 5. (A) Representative chromatograms (detected at 280 mV oxidation potential) and determined apparent BH₄ concentrations for aorta of control and diabetic (STZ) rats. Peak areas detected at the retention time of 5.4 min were converted to a BH₄ concentration using the BH₄ standard curve and was finally normalized to mg protein as estimated by Lowry method in the tissue homogenate supernatant. Data are mean ± SD of the number of indicated independent measurements. Note: * means significantly different to control rats. (B) Representative chromatograms showing co-elution of BH₄ and ascorbate standards using the initial HPLC method (protocol A). (C) Representative chromatograms show clear separation of BH₄ and ascorbate standards using the optimized HPLC method (protocol B).
After optimization of the HPLC method by increasing the pH of the mobile phase (second protocol B: 50 mM potassium phosphate, pH 4.5), we were able to separate the BH₄ signal from the interfering ascorbate peak (Figure 5C). BH₄ was detected at the lower potential of 0 mV and showed a peak at a retention time of 4.56 min, whereas BH₂ was detected at the higher potential of 280 mV and showed a peak at a retention time of 6.92 min (Figure 6A). Standard curves for both BH₄ and BH₂ were linear in the concentration range of 0.3/0.1 to 125/200 µM with R² values of >0.99 (Figure 6B).

Unfortunately, the BH₄ signals in the tissues of control rats were then too low to observe a significant decrease in the tissues of diabetic rats (not shown). We therefore chose to monitor the induction of BH₄ under inflammatory conditions instead, using LPS-induced endotoxemia. We successfully detected BH₄ in the brain, heart and kidney of control and LPS treated rats in the concentration range more similar to previously published data (Table 2). LPS-induced sepsis is known to increase nitric oxide and BH₄ levels via inflammatory pathways via induction of iNOS [19–22]. Chromatogram peaks of BH₄ in the brains were detectable in control and LPS-treated rats (Figure 7A). Heart and kidney levels of BH₄ showed a significant increase in LPS-treated animals as compared to the control, while brain BH₄ concentrations were increased in LPS animals, at least by trend (p-value 0.067) (Figure 7B). In order to prove the authenticity of the potential BH₄ signal in the heart and brain, the samples were spiked with BH₄ standard (Figure 7C).
Table 2. BH$_4$ and BH$_2$ (+biopterin [BP]) levels in different tissues in various animal disease models determined by different methods.

| Species/Strain | BH$_4$ [pmol/mg Protein] | BH$_2$ [pmol/mg Protein] | Reference & Method |
|---------------|--------------------------|--------------------------|--------------------|
| Wistar rats, healthy control and LPS-induced sepsis | Heart: Ctr: 1; 0.3 | n.d. | Present work: HPLC/ECD |
| | LPS: 2; 7.5 | | |
| | Kidney: Ctr: 0.5 | | |
| | LPS: 5.2 | | |
| | Brain: Ctr: 78 | | |
| | LPS: 133 | | |
| Healthy C57BL/CBA mice, GTPCH-I-deficient 4 Hph-1 5 mice | Aorta: Ctr + HFD: 7 | n.d. | [33]: HPLC/ECD |
| | ApoE + HFD: 7-37 | | |
| | Brain: Ctr + HFD: 20-25 | | |
| | ApoE + HFD: 25-30 | | |
| | Endothelial cells Ctr + HFD: 10 | | |
| | ApoE + HFD: 25 | | |
| Healthy C3/HeN mice and septic LPS-treated mice | Aorta: Young: 8 | n.d. | [34]: HPLC/fluorescence with differential oxidation |
| | Old: 5.8 | | |
| Healthy C57BL/6 mice—young versus old | Aorta: Ctr: 100–120 | n.d. | [35]: HPLC/fluorescence with differential oxidation |
| | Hph-1: 50–60 | | |
| C57BL/6 mice and ApoE Tm1Unc mice with Western diet (HFD 6) | Aorta: Ctr + HFD: 7 | n.d. | [36]: HPLC/fluorescence with differential oxidation |
| | ApoE + HFD: 7-37 | | |
| | Brain: Ctr + HFD: 1-2 | | |
| | ApoE + HFD: 1-2 | | |
| C57BL/6 mice and GTPCH-I overexpressing mice (tg-GCH 7) | Aorta: Ctr: 2.7 | n.d. | [37]: HPLC/fluorescence with differential oxidation |
| | tg-GCH: 16 | | |
| New Zealand white rabbits (healthy, hyperglycemic, treatments) | Heart: Ctr: 7.6 | n.d. | [28]: HPLC/ECD |
| | HG 8: 10.2; IPC 9: 14 | | |
| | Ctr + SEP 10: 11; HG + SEP: 6; IPC + SEP: 13 | | |
| | Ctr + DAHP 11: 6; IPC + DAHP: 10 | | |
| Rats, healthy (ZL 12) versus diabetic (ZDF 13) | Kidney: ZL: 6.5 (22 w) | n.d. | [38]: HPLC/ECD |
| | ZLF: 2.5 (22 w) | | |
| | ZDF + ebselen: 6.5 (22 w) | | |
| Rats, healthy (ZL) versus diabetic (ZDF) | Kidney: ZL: <1 (22 w) | n.d. | [39]: LC/MS 15 |
| | ZDF: 0.1 (8 w); ZDF: 1.1 (22 w) | | |
| C57BL/6 mice and DOCA 14 salt hypertension, treatments | Aorta: Ctr: 110; Ctr + BH4: 130 | n.d. | [40]: HPLC/fluorescence with differential oxidation |
| | DOCA: 50; DOCA (p47phox−/−): 90; DOCA (eNOS−/−): 70; DOCA + BH4: 90 | | |
| ApoE−/− mice with oral versus i.v. BH$_4$ administration | Plasma: Peak value (p.o.): 423 nmol/L | n.d. | [41]: LC/MS, derivatization with benzoyl chloride |
| | Peak value (i.v.): 2004 nmol/L | | |
| Healthy volunteers | Plasma: Age of 20: 19.5 nmol/L | n.d. | [42]: LC/MS |
| | Age of 60: 6.6 nmol/L | | |
| Rats, healthy versus ischemia by ligation of the carotid artery | Brain: Sham: 4.5–7 | n.d. | [43]: LC/MS |
| | Ischemia: 5–5.7 | | |
| Diabetic patients with kidney disease | Plasma: Normoalbuminuria: 21.6 nmol/L | n.d. | [44]: LC/MS |
| | Microalbuminuria: 12.9 nmol/L | | |
| | Microalbuminuria: 5.0 nmol/L | | |

1 Ctr, control; 2 LPS, LPS induced sepsis; 3 n.d., not determined; 4 GTPCH-I, GTP-cyclohydrolase-I; 5 Hph-1, hyperphenylalaninemic mouse mutant 90% deficiency GTPCH-I; 6 HFD, high-fat (Western) diet; 7 tg-GCH, endothelial GTPCH transgenic; 8 HG, hyperglycemia; 9 IPC, ischemic preconditioning; 10 SEp, sepiapterin; 11 DAHP, diamino-6-hydroxypyrromidine, an inhibitor of BH$_4$ synthesis; 12 ZL, Zucker nondiabetic lean rat; 13 ZDF, Zucker diabetic fatty rat; 14 DOCA, deoxycorticosterone acetate; 15 LC/MS, liquid chromatography coupled with mass spectrometry.
Figure 7. (A) Representative chromatograms (measured at 0 mV potential) of BH₄ in the brain, heart and kidney of control and LPS-treated septic rats. (B) Concentration levels in these tissues as calculated from BH₄ standard curve. Data are mean ± SD of the number of indicated independent measurements. Note: * means significantly different to control rats. *, p-value is < 0.05; **, p-value is < 0.01 to indicate significance against the control. (C) Representative chromatograms (measured at 0 mV potential) of BH₄ in the heart and brain of control rats with and without spiking with authentic BH₄ standard.

4. Discussion

With the present studies, we systematically investigated the effect of storage conditions and time on stability of BH₄ and BH₂ and established a protocol for their quantification in tissues by using HPLC with electrochemical (coulometric) detection (HPLC/ECD). We showed here that storage conditions play an important role in the stability of BH₄ standards and probably also in tissue samples containing BH₄. Whereas low temperatures in general
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We used DTE and DTPA agents to prevent autoxidation of BH₄ and BH₂. These agents were added to standards, samples and to the mobile phase. In addition to autoxidation in biological samples, reducing agents such as DTE can prevent iron-catalyzed oxidative breakdown of BH₄ [49]. Iron proteins are abundant in cells, leading to continuous oxidation of BH₄ [50]. DTPA is specially effective for preventing redox reactions by metal ions, such as Fe(II)/(III), Mn(II)/(IV) and Cu(I)/(II), that induce oxidative damage by superoxide and hydrogen peroxide [51]. In line with these considerations, the addition of DTE/DTPA largely prevented oxidative degradation of BH₄ standards, even at higher temperature storage conditions: overnight at room temperature or up to two weeks at 4 °C.

LC-MS/MS is mainly used to separate, detect, identify and quantify biomolecules in samples in the presence of complex chemical mixtures. Nevertheless, the HPLC-ECD method performed well in terms of various validation parameters for detection of ascorbate and dihydroartemisinin when compared head to head with LC-MS/MS analysis [52]. Additionally, head-to-head comparison of HPLC/ECD-based quantification of BH₄ or
HPLC/fluorescence detection of neopterin in human plasma of individuals with genetic defects in BH4 and pterin synthesis revealed a small variation from the respective values determined by LC-MS/MS analysis [53]. Whereas the LC-MS/MS method may require more laborious sample preparation, HPLC-ECD requires clear separation of compounds in order to clearly confirm their identity, e.g., by “spiking” with authentic standard. Both techniques have problems with the identification of structural isomers that show similar retention times. LC-MS/MS is a relatively expensive method with respect to the instrument itself as well as maintenance costs. Another major disadvantage of LC-MS/MS is that it works most accurately with volatile buffers. Buffer salts represent a major problem for most LC-MS/MS systems. Direct determination of BH4 in biological samples by optical methods is difficult due to low sensitivity and a lack of fluorescence of BH4 and BH2. Calibration curves for BH4 and BH2 analysis can hardly be found in published work, especially for HPLC with fluorescence or electrochemical detection. Rather old work reported limits of detection for BH4 of 1.24 pmol in 200 µL, corresponding to 6.2 nmol/L when using the differential oxidation and HPLC with fluorescence detection method [43]. For HPLC/ECD, old studies reported limits of detection for BH4 of 0.94 pmol in urine [54] and 0.19 pmol in liver and brain [55]. The combination of electrochemical oxidation and subsequent fluorescence detection allowed measurement with limits of detection for dihydroxanthopterin, 6-biopterin, pterin, BH2 and BH4 of 120, 80, 160, 100 and 60 fmol, respectively [56]. More recent work using the LC-MS/MS method reported a limit of quantitation for BH4 of 10 ng/mL (41.5 nmol/L) in mouse plasma after oral versus intravenous administration of BH4, also allowing kinetic monitoring of the uptake, body distribution and half-life of BH4 [39]. Inaccuracy and imprecision were ≤15% and recovery of BH4 was 80% when samples were stored for 24 h in the presence of DTE [39]. Alternatively, BH4 detection upon derivatization with benzoyl chloride coupled with liquid chromatography–tandem mass spectrometry analysis provided a limit of quantification of 0.02 ng/mL (83 pmol/L) in human plasma, and BH4 levels inversely correlated with age [40]. Other LC/tandem mass spectrometry methods established a limit of quantification of 1 nmol/L for BH4 and BH2 and 2.5 nmol/L for biopterin in cultured endothelial cells [57] and of 1 ng/mL (4.2 nmol/L) for BH4 in human plasma [58]. More discussion of the different analytical methods for BH4 quantification can be found in reference [34].

Oxidation reactions of tetrahydropterins proceed via complex mechanisms to initially give 6H-7,8-dihydro derivatives, which are stabilized by prototrophy to the 7,8-dihydro isomers [59]. Under cellular conditions, BH4 is oxidized to 4a-tetrahydrobiopterin, which is afterwards recycled by 4a-hydroxytetrahydrobiopterin dehydratase or undergoes spontaneous dehydration to 6,7-dihydrobiopterin, and 6,7-BH2 can be reduced back to BH4 by 6,7-dihydropteridine reductase; however, in the absence of sufficient 6,7-dihydropteridine reductase activity, the quinoid 6,7-BH2 rearranges non-enzymatically into the more stable 7,8-BH2 (BH2) [60]. In the system of H2O2-induced oxidation of BH4, we observed the formation of BH2. BH4 synthesis and restoration is mainly based on two important enzymes: GTPCH-I, already mentioned above, and dihydrofolate reductase (DHFR), an enzyme which is responsible for recycling of oxidized BH2 back to BH4 [61,62]. Therefore, they also represent pharmacological targets for the correction of endothelial dysfunction and the prevention of the progression of cardiovascular disease [63,64], as also demonstrated by the prevention of vascular complications in diabetic or hypertensive animals by endothelium-specific overexpression of GTPCH-I [11,65]. As suggested previously, a combination of antioxidant therapy and agents capable of increasing intracellular BH4 levels may be required in order to successfully treat cardiovascular diseases, so precursors of BH4, such as sepiapterin, along with antioxidants, may represent the most promising strategy [64,66].

The initial attempts to measure BH4 levels in diabetic rats and to confirm the reported BH4 decrease failed because of co-elution of BH4 with ascorbate, a known problem, especially since tissues such as the brain are very rich in ascorbate [32,67]. Upon further modification of the HPLC method, we were able to fully separate BH4 and ascorbate peaks.
The main finding of our work is the straightforward detection of BH$_4$ in tissue samples and increased BH$_4$ levels in different organs of septic animals. The BH$_4$ peak identity was confirmed by “spiking” the samples with small amounts of authentic BH$_4$ standard and observing the extent of the increase in the intensity of the peak of interest. In the literature, a large concentration range of BH$_4$ tissue levels were reported, depending on the species, the organ, the health state of the animals and the measurement protocols (see Table 2, more ECD/HPLC examples in [50,68–70]). The BH$_4$ concentrations determined in this report are at the lower range of the published values. Whereas, in human endothelial cells (HAECS and HUVECs), concentrations of BH$_4$ of approximately 0.3 pmol/mg protein were found using an HPLC/ECD method, the level in bovine endothelial cells (BAECs) was 35 pmol/mg protein, and in mouse endothelial cells (sEnd.1) it was 280 pmol/mg protein [50].

The BH$_2$ content in these cells ranged from 2.6 to 120 pmol/mg protein. The strong link between BH$_4$ levels and inflammation (necessary to provide enough BH$_4$ for iNOS activity) was evident from previously reported HPLC/ECD data in septic mice, where plasma BH$_4$ levels increased from 40 to 150 nM within 6 h after induction of sepsis with a subsequent decrease to 70 nM within 24 h after induction of sepsis [22]. In accordance with the changes shown in plasma BH$_4$ concentration, these authors showed that BH$_4$ levels increased from 5 to 8 pmol/mg protein in the heart 6 h after sepsis and were decreased to 3 pmol/mg protein 24 h after starting the experiment. No data on BH$_2$ levels were, however, reported in that study. In another study, the determined concentration of BH$_4$ in the pancreas was 25 pmol/mg protein (80 pmol/mg after BH$_4$ administration) before ischemia and 20 pmol/mg protein (45 pmol/mg after BH$_4$ administration) afterwards [71]. The data presented in Table 2 stresses the large variability in BH$_4$ tissue levels. Of note, it is striking that only two of the reports using the HPLC/ECD method also provided BH$_2$ data for renal tissue that showed at least a 5- or 10-fold increase in diabetic rats and was reduced by ebselen antioxidant therapy to control levels [30,38]. Most published data on tissue BH$_2$ levels are based on the HPLC/fluorescence method with differential oxidation of BH$_4$/BH$_2$, although with only marginal effects of disease or age on BH$_2$ level [35,36] and a striking increase in mice overexpressing the GTPCH-I enzyme [37]. It should be also kept in mind that BH$_2$ data obtained by using the HPLC/fluorescence method with differential oxidation represent a sum of “BH$_2$ plus biopterin” present in the tissue. From these literature data we conclude that BH$_2$ levels are rather low and do not change much in response to many disease conditions when analyzed by the HPLC/ECD method.

As for the biological importance of our findings, BH$_4$ represents a critical regulator of eNOS activity and thereby of endothelial function, determining the vascular tone and anti-adhesive properties of the endothelial cell layer [64]. The oxidative degradation of BH$_4$ as a mediator of eNOS uncoupling is best characterized as a “redox switch” in eNOS activity and supported by studies on hypertension, diabetes, hypercholesterolemia [72] and atherosclerosis [2,9–13]. The depletion of BH$_4$ in almost all cardiovascular diseases is also well established and frequently reviewed [4–8]. The most important enzyme for de novo synthesis of BH$_4$ is GTPCH-I, which was identified as a central regulator of eNOS activity and endothelial function [15], as is also supported by data on eNOS uncoupling and impaired endothelial function in mice with endothelial-specific genetic deletion of GTPCH-I (Gch1<sup>fl/fl</sup>/Tie2cre mice) [16,17]. The ratio of eNOS and GTPCH-I expression levels represents a major determinant of healthy endothelial function, and transgenic overexpression of eNOS without matched elevation of BH$_4$ concentrations ultimately leads to uncoupled eNOS enzyme [12].

Peroxynitrite (ONOO$^-$), a product of a diffusion-controlled reaction between nitric oxide and superoxide [73,74], not only causes oxidative depletion of BH$_4$ but also can induce proteasomal degradation of GTPCH-I by oxidative activation of the 26S proteasome [32,75,76]. In addition to GTPCH-I, DHFR is important for BH$_4$ bioavailability as recycling of oxidized BH$_2$ back to BH$_4$ constitutes the “salvage pathway” [61,62]. The oxidative activation of the 26S proteasome was also shown to be involved in the proteasomal
degradation of DHFR, which was inhibited by eNOS-dependent nitric oxide formation and S-nitrosation of DHFR [77]. Accordingly, the BH₄ system has multiple targets for the prevention of endothelial dysfunction and the mitigation of adverse cardiovascular health effects [63]. In line with the presented data, a combination of antioxidants and BH₄ therapy is recommended to successfully treat endothelial dysfunction and vascular oxidative stress in the setting of cardiovascular diseases [66]. Acute infusion of high local concentrations of BH₄ in smokers [78] and diabetic patients [79] improved endothelial-dependent dilation. Importantly, in vitro, NH₄ scavenged superoxide anion radicals created by the xanthine/xanthine oxidase reaction equipotent to BH₄ but failed to modify acetylcholine-induced changes in forearm blood flow in chronic smokers in vivo, supporting the concept that, in addition to the reactive oxygen species burden of cigarette smoke, a dysfunctional eNOS due to BH₄ depletion may contribute, at least in part, to endothelial dysfunction in chronic smokers [78].

Similarly, administration of the BH₄ precursor folic acid or sepiapterin restored impaired endothelial function in healthy subjects [80,81] as well as in hypertensive and atherosclerotic mice [13,82]. However, despite strong evidence from preclinical and human cohort studies on a therapeutic benefit of eNOS “recoupling” by the administration of BH₄ [78] or its precursors (e.g., sepiapterin or folate) [83], this concept was, until now, not translated to clinical therapy, except for the treatment of phenylketonuria [3]. Therefore, the clinical data are so far inconclusive and warrant larger clinical trials on the cardio/cerebrovascular effects of BH₄-related drugs. Based on the observed instability of BH₄ under oxidative environment, it is also possible that the use of BH₄ precursors may be a more efficient strategy to increase its intracellular levels. Therefore, a sensitive method to monitor BH₄ and its oxidation products in biological/clinical samples is required for establishing its pharmacokinetic properties and to optimize the administration protocol.

5. Conclusions

Our study on standard stability suggests that DTE and DTPA are essential additives for the stabilization of BH₄ under storage conditions, while for BH₂ standards, the addition of HCl is sufficient (HPLC protocol A). With the modified HPLC/ECD method (protocol B) we were able to directly measure BH₄ concentrations in the heart, kidney and brain of control and LPS-treated septic rats. Unfortunately, we were not able to detect BH₂ concentrations in our tissue samples despite clear BH₂ peaks in these preparations upon spiking the sample with authentic BH₂ standard (not shown). We were also not able to establish a significant decrease in BH₄ levels in the aorta of diabetic rats (after separation of BH₄ from ascorbate) but observed a significant increase of BH₄ concentrations in the heart and kidney and also a clear trend of increased BH₄ levels in the brains of septic rats. The data presented herein provide information on BH₄/BH₂ storage conditions and the direct quantification of both biomolecules, especially BH₄ by HPLC/ECD, for researchers interested in the measurement of BH₄/BH₂ in different tissues.

**Author Contributions:** K.V.-M. and A.D. conceived and designed research. K.V.-M., M.O., I.K., M.K. and S.K. carried out experiments; K.V.-M. and A.D. performed data analysis; K.V.-M., J.Z. and A.D. drafted the manuscript; J.Z., H.L., M.O. and T.M. made critical revisions and contributions to the discussion. The work exclusively contains parts of the thesis of K.V.-M. All authors have read and agreed to the published version of the manuscript.

**Funding:** K.V.-M., I.K., M.K. and S.K. hold stipends from the TransMed PhD Program of the University Medical Center Mainz that are funded by the Boehringer Ingelheim Foundation. T.M. is PI of the DZHK (German Center for Cardiovascular Research), Partner Site Rhine-Main, Mainz, Germany. A.D. and T.M. were supported by vascular biology research grants from the Boehringer Ingelheim Foundation for the collaborative research group “Novel and neglected cardiovascular risk factors: molecular mechanisms and therapeutics”.

**Institutional Review Board Statement:** All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health and
approval was granted by the Ethics Committee of the University Medical Center Mainz and the Landesuntersuchungsamt Rheinland-Pfalz (Koblenz, Germany; permit number: 23177-07/G 18-1-001).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are all contained within this article. Raw data are available from the corresponding author upon reasonable request.

Acknowledgments: All data in this manuscript are part of the thesis of K.V.-M.

Conflicts of Interest: The authors declare that they have no conflict of interest with the contents of this article. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

BH2, 7,8-dihydrobiopterin; BH4, tetrahydrobiopterin; CoulArray, coulometric array system; DHFR, dihydrofolate reductase; DTE, 1,4-dithioerythritol; DTPA, diethylenetriaminepentaacetic acid; GTPCH-I, GTP cyclohydrolase I; HPLC/ECD, high performance liquid chromatography with electrochemical detection; LC-MS/MS, liquid chromatography–mass spectrometry; NOS, nitric oxide synthase; STZ, streptozotocin.

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