Kinetics of acid-induced degradation of tetra- and dihydrobiopterin in relation to their relevance as biomarkers of endothelial function

Mortensen, Alan; Lykkesfeldt, Jens

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Introduction
Tetrahydrobiopterin (BH$_4$) is a cofactor of the aromatic amino acid hydroxylases, the nitric oxide synthases (NOS) and the alkylglycerol monooxygenase (Werner et al., 2011). BH$_4$ is easily oxidized to 7,8-dihydrobiopterin (BH$_2$) which may be further oxidized to biopterin. BH$_4$ is essential for the production of nitric oxide, an endothelial vasorelaxing factor, by NOS and may hence serve as a biomarker of endothelial dysfunction. While BH$_2$ may bind to NOS just like BH$_4$ (Vásquez-Vivar et al., 2002; Crabtree et al., 2008), it does not support the formation of nitric oxide (Presta et al., 1998) and instead superoxide is formed (Vásquez-Vivar et al., 2002). Studies have suggested that the ratio of BH$_4$ to BH$_2$ correlates better with endothelial function than the absolute concentration of BH$_4$ (Noguchi et al., 2011; Crabtree et al., 2008; Takeda et al., 2009; Kar & Kavdia, 2011). Consequently, appropriate stabilization and analysis of both BH$_4$ and BH$_2$ is a prerequisite.

BH$_4$ and BH$_2$ in plasma are determined either directly or indirectly by high-performance liquid chromatography (HPLC) employing electrochemical (Powers et al., 1988), fluorescence (Fukushima & Nixon, 1980; Mochizuki et al., 2005) or mass spectrometry detection (Feillet et al., 2008). Prior to analysis plasma proteins are removed; a step most often accomplished by acid precipitation. It has long been known that the auto-oxidation of BH$_4$ proceeds more rapidly at higher pH (Blair & Pearson, 1973; Pearson & Blair, 1975; Pearson, 1974; Berka et al., 2004) and at pH <3 the rate is markedly reduced (Lyudnikova et al., 2009). However, when it comes to BH$_2$, its stability at the low pH used to precipitate proteins is less well-studied. Some authors observed degradation of BH$_2$ in acid (Katoh & Akino, 1966; Schircks et al., 1978) and at neutral pH (Fukushima & Nixon, 1979), whereas others have found a high stability of BH$_2$ in aqueous solution at neutral and acidic pH (Dántola et al., 2008; Maharaj et al., 1990; Heales et al., 1988).

Importantly, however, the previous stability studies of BH$_4$ and BH$_2$ have been performed at micromolar or higher concentration whereas the physiologically relevant concentration range is nanomolar. Also,
the studies have not been performed using the acids commonly used for protein precipitation.

Here, we present the results of a stability study of BH$_4$ and BH$_2$ at nanomolar concentration in the presence of three acids (meta-phosphoric acid (MPA), perchloric acid (PA), and trichloroacetic acid (TCA)) commonly used for plasma protein precipitation. We also report on the stability at neutral pH and examined whether antioxidants may affect the stability of BH$_4$ and BH$_2$ in aqueous solution. Finally, we have studied the stability of biopterins in blood under various clinically relevant conditions.

Methods

Materials

7,8-Dihydro-L-biopterin, 5,6,7,8-tetrahydro-L-biopterin dihydrochloride and L-biopterin were from Shircs Laboratories (Jena, Switzerland). Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), disodium ethylene-diaminetetraacetatedihydrate (EDTA), acetic acid, TCA, citric acid monohydrate, methanol, potassium iodide, hydrochloric acid, sodium hydroxide, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, disodium hydrogen phosphate, dipotassium hydrogen phosphate dihydrate, 1,4-dithioerythritol (DTE), MPA, PA (20%), iodine and sodium dihydrogen phosphate monohydrate were purchased from VWR – Bie&Berntsen A/S (Herlev, Denmark). Ammonium acetate, trisodium citrate dihydrate, 1,4-dithioerythritol (DTE), MPA, PA (20%), iodine and disodium hydrogen phosphate dihydrate were obtained from Sigma-Aldrich (Brøndby, Denmark). All solutions were made in Milli-Q water.

Analysis

The HPLC system consisted of an Agilent (Hørsholm, Denmark) 1100 thermostatted autosampler, an Agilent 1200 binary pump, an Agilent 1200 fluorescence detector (excitation wavelength 275 nm and emission wavelength 350 nm), and an Ultimate 3000 column compartment (excitation wavelength 275 nm and emission wavelength 350 nm). The HPLC system was thermostatted at 35°C and the eluent flow was 1 ml/min.

For the analysis of BH$_4$ and BH$_2$, a Symmetry C8 column (250 × 4.6 mm, 5 μm) from Waters (Hedehusene, Denmark) was used. The samples were eluted with (1) 92% aqueous buffer containing 50 μM EDTA and 50 mM ammonium acetate-acetic acid buffer at pH 5.1 and (2) 8% methanol. The column was thermostatted at 30°C and the eluent flow was 1 ml/min.

A Synergy Polar column (150 × 4.6 mm, 4 μm) and a guard column (4 × 3.0 mm) from Phenomenex (Værløse, Denmark) were used for analysis of blood samples. The samples were eluted with (1) 95% aqueous buffer containing 100 μM EDTA and 50 mM potassium phosphate buffer at pH 6.73 and (2) 5% methanol for 4 min followed by a gradient to 90% B to clean the column. The column was thermostatted at 35°C and the eluent flow was 1 ml/min.

Customarily, 350 nm is used for excitation in biopterin analysis. However, using an excitation wavelength of 275 nm increases the signal around 3 times, thereby increasing the limits of detection and quantitation. The drawback of using 275 nm is the possibility of exciting more compounds, thereby increasing the number of detected compounds and the possibility of overlapping peaks in the chromatogram. With our method the separation of peaks was good when exciting at 275 nm in blood samples and actually did not improve when exciting at 350 nm. Therefore, 275 nm was used throughout.

BH$_4$

A 20 μM stock solution of BH$_4$ in Milli-Q water was stored at –80°C and used within 2 weeks. The stock solution was diluted to 20 nM in 50 mM phosphate buffer at pH 7.4 (with or without DTE or TCEP) or 50 mM citrate buffers at pH 3.0, 4.0, 5.0, and 6.0 (with or without DTE or TCEP). Because TCEP is an acid, the pH of the buffer solutions containing TCEP was adjusted with sodium hydroxide to give the same pH as the buffer without TCEP. Solutions containing 20 nM of BH$_4$ in 5% MPA (with or without DTE or TCEP) or 5% TCA (with or without DTE or TCEP) or 3% PA (with or without DTE or TCEP), which are the commonly used concentrations of acid used for protein precipitation, were also analyzed.

Samples were left in the thermostatted autosampler at 25°C in plastic vials until subjected to analysis. A new vial was used for each injection of 10 μl.

BH$_2$

A 200 μM stock solution of BH$_2$ in Milli-Q water containing either 6.5 mM DTE or TCEP was stored at –80°C and used within 2 weeks. The stock solution was diluted to 20 nM in 50 mM phosphate buffer at pH 7.4 (containing either DTE or TCEP). Because TCEP is an acid, the pH of the buffer solution containing TCEP was adjusted with sodium hydroxide to give the same pH as the buffer without TCEP. Solutions containing 20 nM of BH$_2$ in 5% MPA (containing either DTE or TCEP) or 5% TCA (containing either DTE or TCEP) or 3% PA (containing either DTE or TCEP) were also analyzed. The samples were wrapped in tin foil and stored at 25°C in a thermostatted oven. Aliquots were drawn at intervals and analyzed according to the iodine oxidation method of Fukushima and Nixon (1980) employing the same eluent and column as for the BH$_2$ analysis.

Blood samples

Blood was drawn from a male volunteer in 4 ml K3E BD Vacutainer tubes to which were added 100 μl 4% DTE yielding a final concentration of DTE of around 0.1%. Blood was divided in three aliquots. One aliquot was left in a thermostatic oven at 25°C in the vacutainer. A second aliquot was centrifuged, the plasma separated and placed in the oven. A third aliquot was centrifuged and the plasma divided into three. The three fractions were added TCA, MPA, or PA: to four volumes of plasma was added one volume of 1 M TCA, 25% MPA or 15% PA. The
samples were spun and the supernatant stored in the oven together with the other samples.

Aliquots were taken regularly and analyzed by the method of Fukushima and Nixon (1980). Blood was centrifuged, plasma separated and proteins precipitated with TCA, MPA, or PA before oxidation with iodine. Plasma was added TCA, MPA, or PA.

**Results**

**BH₂**

Regardless of pH, the major detected degradation product of BH₁ was biopterin. Mass balance analysis showed that biopterin could not be the only compound formed from degradation of BH₁ (see below), however, biopterin was the only detectable degradation product of significance. Figure 1A shows the chromatogram of BH₁ in phosphate buffer after 108 h. The peak at 5 min is BH₁ whereas the peak at 5.36 min is biopterin. A number of very small peaks could also be discerned. It was not the purpose of this study to precisely elucidate the reaction mechanisms and determine all degradation products.

Degradation of BH₁ followed first-order kinetics, i.e. exponential loss of BH₁ in all solutions examined. The first-order rate constants are presented in Table 1. These rate constants are in reality not true first-order rate constants, as they depend on pH and may also depend on oxygen concentration (see Discussion section). However, the concentration of hydrogen ions can be considered to be constant over time in the solutions, either because the solutions are buffered or because of a large surplus of hydrogen ions compared to BH₂ in the acidic solutions.

Likewise, the concentration of oxygen is much higher than the concentration of BH₂ and can be considered to remain constant throughout the course of reaction as well.

BH₁ is rather stable in solution with half-lives of >2.5 h regardless of pH. However, pH is not the only determinant of BH₁ stability. Somewhat surprisingly, the rate of degradation of BH₁ is lower in PA than in TCA and MPA, despite the lower pH and despite the fact that PA, besides being a strong acid, is also an oxidizing acid that potentially could oxidize BH₁. The rate of degradation is also higher in MPA than in TCA despite the higher pH in the former. Comparing the phosphate buffer at pH 7.4 and the citrate buffer at pH 6.0, the higher pH of the phosphate buffer leads to an increase in reaction rate rather than a decrease.

From the rate constants in Table 1, it is clear that the rate of degradation of BH₁ is not proportional to the hydrogen ion concentration, but the (apparent) first-order rate constant shows a weaker dependence on acidity. Looking at the citrate buffers alone, the first-order rate constants could be shown to perfectly follow a second-order polynomial expression in the pH range 3–6:

\[
k = 0.01 \times (\text{pH})^2 - 0.12 \times \text{pH} + 0.38 \quad (\text{BH}_1 \text{alone})
\]

\[
k = 0.0055 \times (\text{pH})^2 - 0.076 \times \text{pH} + 0.26 \quad (\text{BH}_1 \text{with TCEP})
\]

\[
k = 0.0059 \times (\text{pH})^2 - 0.086 \times \text{pH} + 0.31 \quad (\text{BH}_1 \text{with DTE})
\]

These mathematical relationships indicate that the mechanism of degradation is not merely a matter of protonation of BH₁, followed by oxidation of the protonated BH⁺, the pKₐ of which has been determined to be 2.56 (Maharaj et al., 1990).

Since the major pathway of degradation of BH₁ in neutral and acidic solution is oxidation, it is relevant to examine if antioxidants could stabilize BH₁. DTE and the closely related dithiothreitol (DTT) are often used to stabilize BH₄ in plasma before analysis. A concentration of 0.1%, corresponding to 6.5 mM, is commonly employed at which concentration DTT has been found to be able to stabilize BH₄ in plasma for a few hours (Fekkes & Voskuilen-Kooijman, 2007). TCEP is an antioxidant used primarily for reducing disulfide bonds. However, it has been successfully used for stabilizing ascorbic acid in solution (Lykkesfeldt, 2000) and we therefore decided to test its suitability with respect to biopterins. The same concentration, i.e. 6.5 mM, of TCEP was used throughout.

Neither of the two antioxidants was very effective in protecting BH₁ from oxidation (Table 1). In solutions of the three acids, both antioxidants conferred the same level of modest protection to BH₁. As far as DTE goes, this is somewhat surprising, as the antioxidative capability is considered to be lost at low pH. In citrate and phosphate buffers, TCEP does provide some protection whereas DTE does not. In fact, DTE seems to have a pro-oxidant effect at
pH 4.5, and 7.4 (Table 1). Besides affecting the rate of degradation of BH2, DTE also affected the reaction pathway. In Figure 1B it can be seen that DTE caused formation of a compound eluting at 4.73 min. This compound was observed in citrate and phosphate buffers but not in the more acidic solutions. It was virtually absent in solutions of BH2 without antioxidants and in solutions with TCEP. The nature of this compound remains to be established.

At pH 7.4 after 108 h of autoxidation, BH2 and biopterin accounted for 100% of the added BH2. With TCEP, BH2, and biopterin also accounted for the majority of species present (around 94%) after 108 h. However, in the presence of DTE at pH 7.4, the concentration of BH2 and biopterin only amounted to 30% each, that is, 40% of the initial amount of BH2 had been converted to compound(s) other than biopterin, one of them the species at 4.73 min. In general, regardless of pH more BH2 could be recovered as either BH2 or biopterin at the end of the reaction if no antioxidant was used, whereas DTE gave a low recovery of BH2 as either BH2 or biopterin; TCEP was in between (Table 2).

BH4

The stability of BH4 in the three acids was also studied. Because of its instability, BH4 stability was only examined in the presence of antioxidant—in clinical settings, DTE or DTT is routinely added to the sample to avoid degradation.

BH4 was remarkably stable in all three acids with DTE or TCEP. Over a period of 48 h no statistically significant degradation took place with one exception: BH4 in TCA with DTE (p < 0.001, linear regression) (Figure 2). From the data, it was not evident whether the degradation was zero- or first-order: zero-order rate constant 0.094 ± 0.006 nM/h (R2 = 0.977) or first-order rate constant 0.0053 ± 0.0004 h⁻¹ (R2 = 0.973). Concomitantly, the level of oxidized biopterins (BH2 and biopterin) rose. Another interesting finding was that the response of BH4 was around 20% lower in MPA than in TCA and PA, regardless of whether DTE or TCEP was used. This lower response did not seem to be due to degradation as the concentration of BH4 in MPA did not change with time (Figure 2).

The stability of BH4 in phosphate buffer at pH 7.4 was also studied. In Figure 3 is shown the degradation of BH4 as a function of time. In contrast to the situation at low pH, BH4 stability at pH 7.4 was markedly reduced. Apparently, the degradation of BH4 with DTE followed zero-order kinetics with a rate constant of 0.254 ± 0.013 nM/h, while

### Table 1. First-order rate constants (h⁻¹) for degradation of BH2.

<table>
<thead>
<tr>
<th>Acid/buffer</th>
<th>pH</th>
<th>BH2</th>
<th>BH2 + TCEP</th>
<th>BH2 + DTE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>0.75</td>
<td>0.0943 ± 0.0022</td>
<td>0.0786 ± 0.0011</td>
<td>0.0828 ± 0.0013</td>
</tr>
<tr>
<td>TCA</td>
<td>0.89</td>
<td>0.2112 ± 0.0038</td>
<td>0.1475 ± 0.0013</td>
<td>0.1464 ± 0.0011</td>
</tr>
<tr>
<td>MPA</td>
<td>1.51</td>
<td>0.2701 ± 0.0058</td>
<td>0.2434 ± 0.0023</td>
<td>0.2441 ± 0.0017</td>
</tr>
<tr>
<td>Citrate</td>
<td>3.0</td>
<td>0.1050 ± 0.0034</td>
<td>0.0814 ± 0.0004</td>
<td>0.1037 ± 0.0007</td>
</tr>
<tr>
<td>Citrate</td>
<td>4.0</td>
<td>0.0496 ± 0.0011</td>
<td>0.0454 ± 0.0005</td>
<td>0.0587 ± 0.0007</td>
</tr>
<tr>
<td>Citrate</td>
<td>5.0</td>
<td>0.0216 ± 0.0009</td>
<td>0.0170 ± 0.0003</td>
<td>0.0273 ± 0.0004</td>
</tr>
<tr>
<td>Citrate</td>
<td>6.0</td>
<td>0.0061 ± 0.0005</td>
<td>0.0028 ± 0.0003</td>
<td>0.0060 ± 0.0003</td>
</tr>
<tr>
<td>Phosphate</td>
<td>7.4</td>
<td>0.0087 ± 0.0008</td>
<td>0.0050 ± 0.0003</td>
<td>0.0112 ± 0.0003</td>
</tr>
</tbody>
</table>

BH2, dihydrobiopterin; DTE, 1,4-dithioerythritol; TCEP, tris(2-carboxyethyl)phosphine hydrochloride.

### Table 2. Percent recovery of BH2 as either BH2 or biopterin at the end of the degradation.

<table>
<thead>
<tr>
<th>Without antioxidant</th>
<th>TCEP</th>
<th>DTE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>71</td>
<td>67</td>
</tr>
<tr>
<td>TCA</td>
<td>81</td>
<td>66</td>
</tr>
<tr>
<td>MPA</td>
<td>54</td>
<td>43</td>
</tr>
<tr>
<td>Citrate pH 3</td>
<td>69</td>
<td>61</td>
</tr>
<tr>
<td>Citrate pH 4</td>
<td>79</td>
<td>70</td>
</tr>
<tr>
<td>Citrate pH 5</td>
<td>86</td>
<td>80</td>
</tr>
<tr>
<td>Citrate pH 6</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>Phosphate pH 7.4</td>
<td>100</td>
<td>94</td>
</tr>
</tbody>
</table>

BH2, dihydrobiopterin; DTE, 1,4-dithioerythritol; MPA, metaphosphoric acid; PA, perchloric acid; TCA, trichloroacetic acid; TCEP, tris(2-carboxyethyl)phosphine hydrochloride.
the degradation in the presence of TCEP was first-order with a rate-constant of 0.145 ± 0.025 h⁻¹.

**Blood**

Stability of blood was only studied with DTE as the prior experiments with BH₄ had indicated that DTE was a better antioxidant than TCEP at pH 7.4. Regardless in which state blood samples were left at 25°C (whole blood, plasma or acidified plasma) and which acid was used for precipitation, no significant changes in BH₄ or BH₂ levels or the BH₂-to-BH₄ ratio over time were found. However, there were considerable differences in analytical response or recovery. The data were analyzed by two-way ANOVA using the acid and pretreatment of the blood sample as factors followed by Tukey’s *post hoc* test of planned comparisons. Table 3 shows the results with respect to acid used for precipitation. BH₂ levels were ~30–35% lower when precipitating proteins with MPA compared to the two other acids. TCA gave a slightly higher BH₄ level than did PA. The situation was reversed when it came to the oxidized biopterins BH₂ and biopterin: here MPA gave the highest level and TCA the lowest level. Despite the higher level of BH₂ + biopterin with MPA, it was not enough to offset the lower level of BH₄ and the total level of biopterins was significantly reduced compared to the two other acids; there was no difference between TCA and PA (Table 3).

When it comes to pretreatment of the samples, BH₄ levels were unaffected by how the blood samples were stored (Table 4). BH₂ + biopterin levels, however, were slightly lower in the acid-precipitated samples than in whole blood and plasma samples. This affected the BH₂-to-BH₄ ratio but not the level of total biopterins.

**Discussion**

In the present study, we wanted to investigate the stability of BH₄ and BH₂ both under controlled aqueous conditions and more clinically relevant conditions in order to provide a rational basis for recommending a standard stabilization procedure which adequately preserves the *in vivo* equilibrium of the analytes.

With regard to BH₄, the present study demonstrates—in line with previous findings—that BH₄ is degraded in aqueous solution at neutral and acidic pH, the major degradation product being biopterin. Katoh and Akino observed that BH₂ in 0.5 N sulfuric acid (stored overnight at –20°C) was oxidized to biopterin and to a lesser extent dehydroxylated to form deoxysepiapterin (Katoh & Akino, 1966). Likewise, BH₂ in 25% acetic acid was found to be degraded to a number of compounds, the major compound being biopterin even when the reaction was carried out under nitrogen for 20 min at 60°C (Schircks et al., 1978). Formation of biopterin was believed to be due to intermolecular redox reactions. Fukushima and Nixon (1979) studied the autoxidation of BH₂ at pH 4, 6, and 7.5. After 2–3 days of autoxidation at room temperature they found that biopterin was the major product with minor amounts of sepiapterin, deoxysepiapterin, and xanthopterin. None of these studies looked at the kinetics of degradation of BH₂.

However, other studies failed to observe any degradation. Thus, Maharaj et al. found that the absorption spectrum of BH₂ did not change with time (time span not indicated) at low pH (0.15 N and 5 N HCl) indicating that BH₂ was stable under these conditions (Maharaj et al., 1990). The lack of any observable changes may have been due to a too short period of observation. Likewise,

Figure 3. Degradation of tetrahydrobiopterin (BH₄) in phosphate buffer (pH 7.4) with dithioerythritol (DTE) (closed circle) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (closed square).

Table 3. Plasma levels of biopterins with respect to acidification strategy.

<table>
<thead>
<tr>
<th>BH₄/nM</th>
<th>BH₂ + Biopterin/nM</th>
<th>Total/nM</th>
<th>BH₂–BH₄ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA</td>
<td>24.9 ± 1.0*</td>
<td>9.2 ± 1.3*</td>
<td>34.1 ± 1.0*</td>
</tr>
<tr>
<td>MPA</td>
<td>16.3 ± 1.3***</td>
<td>12.6 ± 1.3***</td>
<td>28.9 ± 1.5***</td>
</tr>
<tr>
<td>PA</td>
<td>23.1 ± 1.1***</td>
<td>10.2 ± 0.9*</td>
<td>33.3 ± 0.9*</td>
</tr>
</tbody>
</table>

Numbers bearing different superscript lettering are significantly different. BH₂, dihydrobiopterin; BH₄, tetrahydrobiopterin; MPA, meta-phosphoric acid; PA, perchloric acid; TCA, trichloroacetic acid. *p < 0.05, ***p < 0.001 compared to TCA.

Table 4. Bench stability (25°C) of biopterins under various conditions.

<table>
<thead>
<tr>
<th>BH₄/nM</th>
<th>BH₂ + Biopterin/nM</th>
<th>Total/nM</th>
<th>BH₂–BH₄ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>21.0 ± 4.0</td>
<td>11.0 ± 2.1*</td>
<td>32.1 ± 2.3</td>
</tr>
<tr>
<td>Plasma</td>
<td>21.5 ± 3.9</td>
<td>11.0 ± 1.6*</td>
<td>32.4 ± 2.6</td>
</tr>
<tr>
<td>Acid-precipitated</td>
<td>21.7 ± 4.0</td>
<td>10.0 ± 1.7*</td>
<td>31.8 ± 3.0</td>
</tr>
</tbody>
</table>

Numbers within a column bearing different superscript lettering are significantly different. BH₂, dihydrobiopterin; BH₄, tetrahydrobiopterin. *p < 0.05.
Heales et al. did not observe any degradation of BH$_4$ at pH 1.6, 5.3, and 7.6 when autoxidized for 30 min (Heales et al., 1988). As shown here, BH$_4$ is only degraded to a small extent in 30 min. Davis et al. observed no degradation of BH$_4$ for up to 150 min at pH 6.8–8.2 (Davis et al., 1988), which is in line with our results showing little degradation during this short-time span. On the other hand, Dántola et al. followed the degradation of BH$_4$ at neutral pH at 25°C for 2 days and observed very little degradation (Dántola et al., 2008). They determined a first-order rate constant of degradation of BH$_4$ of 0.0004 h$^{-1}$ (this rate constant should be regarded with some caution as the reaction was only followed for 48 h, during which very little reaction would have taken place), which is at least an order of magnitude lower than our results at pH 6.0 and 7.4. They did not use buffers but adjusted pH to 7.0 using hydrochloric acid or aqueous sodium hydroxide. In combination with our results, this could indicate that buffers actually enhance the degradation of BH$_4$, in line with our observations that the rate of degradation depends on the nature of the buffer/acid and not only on pH. That the nature of the buffer also plays a role in the degradation of BH$_4$ (and BH$_2$), has been demonstrated previously where it was found, that the degradation product profile was different in succinate, phosphate and Tris buffer at the same pH (Fukushima & Nixon, 1979).

With regard to BH$_2$, our results showed an increased stability of BH$_2$ at low pH compared to neutral pH, which is in line with previous findings. Autoxidation of BH$_2$ proceeded more rapidly as pH was increased (Blair & Pearson, 1973; Pearson & Blair, 1975; Pearson, 1974). At high pH, this is due to deprotonation of the uncharged BH$_2$ (pK$_a$ 10.5 (Blair & Pearson, 1973)) leading to a negatively charged species that is easily oxidized. On the other hand, at lower pH singly (pK$_a$ 5.6 (Pfleiderer, 1982)) or even doubly charged species (pK$_a$ 1.3 (Pfleiderer, 1982)) are formed which are less susceptible toward oxidation. Thus, at pH <3 the rate was markedly reduced and at pH 1–2 no degradation was observed during 20-min incubation (Lyudnikova et al., 2009). On the other hand, Howells and Hyland observed that 41 nM BH$_2$ in 0.1 M HCl (pH around 1) was completely oxidized to BH$_2$ and biopterin in 15 min (Howells & Hyland, 1987). Increasing the concentration to 415 nM meant that only 1.3% was oxidized in 15 min, and increasing the concentration even further led to an even lower percentage being oxidized (Howells & Hyland, 1987), showing that results at micromolar (in the study by Lyudnikova et al. a concentration of around 0.1 mM was employed) and millimolar concentrations cannot be used to predict the behavior at the physiologically relevant nanomolar range.

At higher pH, the oxidation of BH$_2$ is faster. The half-life of BH$_2$, can be estimated to be around half an hour at pH 3 and at pH 4 and above almost complete oxidation takes place in 20 min (Lyudnikova et al., 2009). At pH 5.3–7.6 the half-life was estimated to around 9 min (Heales et al., 1988), and another study found half-lives of 6.5–60 min at pH 6.8–8.2 depending on the buffer used (Davis et al., 1988). Similarly, a half-life of 15–20 min was observed at pH 7.4 (Valent & Tóth, 2006). Somewhat greater stability was found by Berka et al. (2004) who observed half-lives of 40 and 120 min at pH 7 and 3, respectively.

BH$_4$, being more reduced than BH$_2$, is more prone to oxidation than BH$_2$, and an antioxidant is almost always used when handling samples containing BH$_4$. Co-administration of antioxidants greatly enhances the stability of BH$_4$. Our studies showed no significant degradation of BH$_4$ in acid with either DTE or TCEP for 48 h except for the combination TCA and DTE. Why BH$_4$ showed less stability in TCA with DTE than in the two other acids is not known. A previous study has shown that acid alone was not enough to stabilize BH$_4$ at nanomolar concentration (Howells & Hyland, 1987), whereas at micromolar concentration acid without antioxidant was sufficient to stabilize BH$_4$ at least for 20 min (Howells & Hyland, 1987; Lyudnikova et al., 2009). Ascorbic acid (5.7 mM) prevented oxidation of 31 μM BH$_4$ for 20 h and 41.5 nM BH$_4$ (both containing 0.1 M HCl) for 4 h when the solutions were gassed with argon (Howells & Hyland, 1987). In another study, it was found that 100 nM BH$_4$ at pH 7.4 was completely oxidized within 20 min and that 100 μM ascorbic acid increased the half-life to around 70 min (Heller et al., 2001). Berka et al. found that DTT was able to lower the decay rate fourfold at pH 7, and that DTE together with DETAPAC was able to increase the half-life by a factor of 6 at pH 3 (Berka et al., 2004). Another study found that 0.5 mM DTT inhibited the oxidation of BH$_4$ at pH 7.4 by 85–90% (Valent & Tóth, 2006). Other studies have found that 0.1% DTE or 0.1% DTT is able to stabilize BH$_4$ for 2.5–5 h when a chelating agent such as EDTA is also used (Cañada-Cañada et al., 2009; Espinosa-Mansilla et al., 2008; Powers et al., 1988; Howells et al., 1986). Our studies showed that degradation of BH$_4$ at pH 7.4 begins immediately and that there is not a lag phase during which no degradation takes place (Figure 3), as other studies might indicate. However, with a rate constant of 0.25 mM h$^{-1}$ in DTE only 1% of BH$_4$ would have degraded during the first hour.

The lower BH$_4$ response with MPA remains unclear. It does not seem to be degradation as there is no change for 50 h (Figure 2), but interference with the assay, i.e. oxidation by iodine, cannot be ruled out.

Only few publications have reported on the stability of biopterins in blood samples. Fiege et al. studied the stability of BH$_4$ in blood samples stored at −80°C for 8 months. They found that without antioxidant all BH$_4$ was oxidized and that DTE was better at stabilizing BH$_4$ than ascorbate (Fiege et al., 2004). Another study examined the stability of BH$_4$ in blood at room temperature (Fekkes & Voskuilen-Kooijman, 2007). It was found that 0.1% DTT offered better protection than 0.5 and 0.25% DTT. It was also observed that centrifugation of the blood sample right after it had been collected gave a lower total biopterin level compared to waiting for 3 h before centrifugation of the blood sample and after 3 h the level of BH$_4$ started to decline whereas the total biopterin level remained constant for
24 h (Fekkes & Voskuilen-Kooijman, 2007). Our results do not show a higher biopterin level if the blood sample is allowed to stand for 3 h prior to centrifugation. However, if the methodology by Fekkes and Voskuilen-Kooijman is correctly described in their paper, it appears that the authors did not stoichiometrically add enough iodine to oxidize the added DTT. This could explain their findings since enough of the DTT could have reacted after 3 hours to leave sufficient iodine to oxidize BH₄ and BH₂ and thereby give a higher response.

Some of the observations that were made in the BH₄ and BH₂ model systems were also found in the blood samples whereas others were not. BH₂ in model systems was degraded by acid whereas this did not seem to be the case in blood. The iodine oxidation method gives the total amount of BH₄ and biopterin and if BH₂ were converted exclusively to biopterin, this would not affect the measured amount. However, the model studies with BH₄ clearly showed (Table 2) that BH₄ is not only converted to biopterin but also to some unidentified species. Hence, if BH₂ were degraded by acid in blood a lowering of the BH₂ biopterin level would have been expected. As this is not the case, protective factors such as endogenous ascorbate or EDTA coming from the blood sampling tubes are more likely to explain the findings. BH₂ was found to degrade in model systems at pH 7.4 (Figure 3). This was not reproduced in blood samples. Even though degradation is slow (0.25 nM/h) about 1.5 nM, corresponding to 6%, would have degraded in 6 h which would have been detectable. As above, the most likely reason is the presence of stabilizing factors in the blood samples such as ascorbate and EDTA. One significant finding that was observed in both model systems and blood samples was the lower level of BH₄ with MPA, which seemed to be even worse in blood samples than in the model systems, and the lower recovery of total biopterins in blood samples (Table 3) and the BH₄ model system (Table 2). TCA provided the highest level of BH₄ and lowest level of BH₂.

The reason for the differences in BH₄ and BH₂ levels observed with the three acids is not clear. The inverse relationship between BH₄ and BH₂ levels observed for the three acids (Table 3) could indicate different degrees of oxidation of BH₂, i.e. most oxidation with MPA and least with TCA. However, levels of BH₂ and BH₄ did not change with time, which would have been expected if oxidation took place. Furthermore, the model studies showed that BH₂ was not oxidized for 48 h in acid (with the exception of TCA with DTE). It therefore seems more likely that the acids interact differently with the iodine oxidation assay. MPA is unsuitable for biopterin analysis with the iodine oxidation assay, as the levels of BH₂ are simply too low. Presumably, a higher BH₂ level reflects less post-sampling oxidation, which leaves TCA the better choice as the level of BH₂ is significantly higher and the level of total biopterins the same compared to PA (Table 3).

Comparing the different pretreatments of the samples, acid precipitation of the blood sample immediately after blood drawing seems to give the lowest level of BH₂ and hence least oxidation (Table 4). However, the differences are small, albeit significant, and no method seems to be vastly superior to the others.

**Conclusions**

The high stability of BH₄ and degradation of BH₂ in acid and vice versa at neutral pH poses an analytical challenge when using the combined measurement as a biomarker of endothelial function. However, we have shown that the levels of BH₂+ biopterin and BH₂ did not change with time for up to 6.5 h. TCA is the best choice for precipitating proteins whereas MPA should not be used. Whether plasma is separated and proteins precipitated immediately or at a later stage only has a minor effect on the levels of biopterins determined.

**Declaration of interest**

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**References**


