Published in final edited form as:

Arch Biochem Biophys. 2012 April 1; 520(1): 7–16. doi:10.1016/j.abb.2012.01.010.

HPLC analysis of tetrahydrobiopterin and its pteridine derivatives using sequential electrochemical and fluorimetric detection: application to tetrahydrobiopterin autoxidation and chemical oxidation

Roberto Biondi^{*,+,#,†}, Giuseppe Ambrosio^{*,†}, Francesco De Pascali⁺, Isabella Tritto^{*,†}, Enrico Capodicasa[†], Lawrence J. Druhan^{+,‡}, Craig Hemann⁺, and Jay L. Zweier⁺

^{*}Division of Cardiology, University of Perugia School of Medicine, 06100 Perugia

⁺Davis Heart and Lung Research Institute, Division of Cardiovascular Medicine, Department of Internal Medicine, College of Medicine, Ohio State University, Columbus, OH 43210

[‡]Department of Anesthesiology, College of Medicine, Ohio State University, Columbus, OH 43210

[#]Azienda Ospedaliera "S.Maria" Terni

[†]Dipartimento di Medicina Clinica Sperimentale, University of Perugia School of Medicine, 06100 Perugia

Abstract

Tetrahydrobiopterin (BH₄) is an essential cofactor of endothelial nitric oxide (NO) synthase and when depleted, endothelial dysfunction results with decreased production of NO. BH₄ is also an anti-oxidant being a good "scavenger" of oxidative species. NADPH oxidase, xanthine oxidase, and mitochondrial enzymes producing reactive oxygen species (ROS) can induce elevated oxidant stress and cause BH₄ oxidation and subsequent decrease in NO production and bioavailability. In order to define the process of ROS-mediated BH₄ degradation, a sensitive method for monitoring pteridine redox-state changes is required. Considering that the conventional fluorescence method is an indirect method requiring conversion of all pteridines to oxidized forms, it would be beneficial to use a rapid quantitative assay for the individual detection of BH₄ and its related pteridine metabolites. To study, in detail, the BH₄ oxidative pathways, a rapid direct sensitive HPLC assay of BH₄ and its pteridine derivatives was adapted using sequential electrochemical and fluorimetric detection. We examined BH₄ autoxidation, hydrogen peroxide- and superoxide-driven oxidation, and Fenton reaction hydroxyl radical-driven BH₄ transformation. We demonstrate that the formation of the primary two-electron oxidation product, dihydrobiopterin (BH₂), predominates with oxygen-induced BH₄ autoxidation and superoxide-catalyzed oxidation, while the irreversible metabolites, pterin and dihydroxanthopterin (XH₂), are largely produced during hydroxyl radical-driven BH₄ oxidation.

© 2012 Elsevier Inc. All rights reserved.

To whom correspondence should be addressed: Prof. Jay L. Zweier, Davis Heart & Lung Research Institute, The Ohio State University Medical Center, 473 W. 12th Avenue, Suite 611, Columbus, Ohio 43210, 614-247-7788 Phone, 614-247-7845 Fax, Jay.Zweier@osumc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

nitric oxide; superoxide; nitric oxide synthase; hydroxyl radical; reactive oxygen species; Fenton reaction

INTRODUCTION

Tetrahydrobiopterin (BH_4), a molecule belonging to the pteridine family, is an essential cofactor for the aromatic amino acid hydroxylases (i.e. phenylalanine, tyrosine and tryptophan hydroxylase), which are key enzymes in the biosynthesis of several neurotransmitters, including catecholamines and serotonin [1, 2]. Another BH_4 -dependent enzyme, alkylglycerol monooxygenase (glyceryl-ether monooxygenase), cleaves the O-alkyl bond of ether lipids. These lipids are important in spermatogenesis, cell signaling, brain membrane structure and protection against cataracts [3]. In the skin depigmentation disorder, vitiligo, the recycling process of BH_4 is disrupted due to the high levels of hydrogen peroxide which accumulate in the epidermis, deactivating the enzyme 4a-OH- BH_4 dehydratase. This leads to accumulation of both 6- and 7-biopterin, imparting a characteristic fluorescence to the skin with UVA (351 nm) illumination. Photolysis of both sepiapterin and 6-biopterin in the epidermis leads to the production of pterin-6-carboxylic acid and hydrogen peroxide [4, 5].

In addition, in more recent years, BH_4 has been shown to be an essential cofactor of nitric oxide synthase (NOS), involved in the endothelium-dependent control of vascular function by supporting nitric oxide (NO) production [6]. While the exact role of BH_4 in NO catalysis is still debated, the mechanisms by which BH_4 affects NOS catalysis include the stabilization of the enzyme active conformation, acting as an allosteric effector that enhances the substrate binding, and potentially functioning as a posttranscriptional stabilizer of NOS mRNA [2, 7]. Moreover, BH_4 is involved in the formation of the activated oxo-heme moiety that is necessary for the monooxygenation of substrate necessary for NOS catalytic activity [8]. In addition, BH_4 is a potent antioxidant and a scavenger for oxygen-derived free radicals [2, 9]. As such, BH_4 is an essential NOS cofactor, and when BH_4 levels fall to values that are insufficient to saturate eNOS, this contributes to endothelial dysfunction with decreased production of NO.

This endothelial dysfunction is considered a prognostic marker of cardiovascular diseases [10], including hypercholesterolemia, diabetes, atherosclerosis, hypertension and heart failure [11–15]. Whereas endothelial dysfunction is likely a multifactorial process [16], there is now much data suggesting that increased vascular production of reactive oxygen species (ROS) plays an important role [11, 17]. In fact excessive ROS production has been found in coronary arteries in patients with coronary disease [18]. Moreover, superoxide anion (O_2^-) reacts rapidly with NO, resulting in the formation of peroxynitrite and loss of NO bioavailability. Furthermore, it has been demonstrated that increased vascular ROS production promotes the oxidative degradation of BH_4 leading to eNOS “uncoupling” with reduced NO production from the enzyme [19–22]. In the blood vessel, ROS are produced by several enzymes, including xanthine oxidase (XO), cyclooxygenase and NADPH oxidase. Moreover, *in vitro* biochemical studies demonstrated that NOS can independently produce O_2^- when BH_4 bioavailability is reduced [23]. Thus, in the presence of a suboptimal concentration of BH_4 , NOS activation leads to O_2^- production from the “uncoupled” enzyme.

A close link between O_2^- production and BH_4 depletion with increased levels of dihydrobiopterin (BH_2) and biopterin (B) has been demonstrated in endothelial cells from

diabetic biobreeding (BB) rats [24] and in the aortas from an insulin resistant rat model induced on a high-fructose diet [25]. In addition, BH₄ oxidation has been demonstrated in vessels from mice with deoxycorticosterone (DOCA)-salt hypertension [15] and in the aortas from diet-induced hypercholesterolemic rabbits [20]. From examination of these experiments, it was clear that elevated oxidative stress caused enhanced BH₄ oxidation, and, consequently, vascular tissue levels of BH₂ and B increased. Moreover, it was proposed that the oxidative stress produced “uncoupling” of eNOS with a decrease of endothelial NO bioavailability, not only due to decreased BH₄ levels, but also because of an increased ratio of BH₂/BH₄ [19]. However, impaired endothelial function with irreversible BH₄ oxidation was observed in the ischemic heart. The BH₄ degradation was likely caused by the oxidative effect of oxygen free radicals that are enhanced during myocardial ischemia. The observed loss of BH₄ in the ischemic heart was accompanied by a rise in the metabolite dihydroxanthopterin (XH₂) which cannot be enzymatically converted back to BH₄, and no formation of the potentially reversible oxidation product BH₂ was detected [26].

In the present paper, a sensitive method is described and validated for direct measurement of BH₄ and its main metabolites, BH₂, B, pterin (P) and XH₂, with sequential electrochemical and fluorimetric detection [27]. This method has major advantages over the commonly used indirect method in which BH₂ and BH₄ are oxidized to the fully aromatic form, B, by iodine and then detected by fluorescence HPLC; along with differential oxidation using acidic and alkaline pH medium in order to calculate the BH₄ and BH₂ concentrations [28]. With direct detection, each related compound can be directly quantitated providing more specific information on each, eliminating the need for separate reactions. We utilized the direct detection method to investigate the action of oxidative stress on BH₄ degradation with particular regard to the identification and quantitation of the oxidized forms of BH₄ produced from different oxidants. An HPLC method that utilizes post-separation electrochemical oxidation and subsequent fluorescence determination was developed and utilized as modified from the approach described by Hyland [27]. This assay can be used to clarify, through the evaluation of the individual BH₄ pteridine derivatives, how BH₄ catabolism is affected in a variety of vascular dysfunctions whose pathogenesis is ROS related.

MATERIALS AND METHODS

Reagents

Acetonitrile (ACN), iron(II) sulfate heptahydrate, citric acid, octyl sulphate sodium salt (OSA), diethylenetriaminepentaacetic acid (DTPA), DL-Dithiothreitol (DTT), nitrilotriacetic acid (nta), dimethylthiourea (DMTU), dimethyl sulfoxide (DMSO) anhydrous, (±)- α -Tocopherol phosphate disodium salt (Trolox), catalase from bovine liver (CAT), superoxide dismutase from bovine liver (SOD), potassium dioxide (KO₂), cytochrome *c* from equine heart and ascorbic acid were from Sigma (St. Louis, MO). Tetrahydro-L-biopterin hydrochloride (BH₄) and 7,8 dihydro-L-biopterin (BH₂) were from Cayman Chemical and dihydroxanthopterin (XH₂), pterin (P) and 6-biopterin (B) were from Schircks Lab (Jone, Switzerland). All other reagents used in this work were from Sigma (St. Louis, MO).

HPLC analysis of pteridines

The HPLC analysis of pteridines was carried out using a Waters Atlantis dC-18 5 μ M reverse phase column (4.6 $\times$ 100 mm) with flow rate set at 1.3 ml/min and an isocratic elution consisting of 6.5 mM NaH₂PO₄, 6 mM citric acid, 1 mM OSA, 2.5 mM DTPA, 1 mM DTT and 2% ACN, pH=3.0. The OSA was used in the mobile phase as an ion-pairing reagent which acts as an anionic counterion for the separation and resolution of positively

charged analytes [29]. The DTPA was added to chelate transition metals to prevent oxidation of the analytes, and the DTT was used as a reductant to further stabilize the reduced forms of the pteridines [26, 27, 30]. A block diagram of the instrument configuration is shown in Figure 1. The HPLC system as purchased from ESA, Inc. is comprised of a Model 5600A CoulArray System controlled by the CoulArray Data Station Program V3.10 to coordinate all system modules including a Model 584 Solvent Delivery Module, a Model 542 Autosampler, a Model 5020 Guard Cell, and both the electrochemical and fluorescence detectors. The Model 6210 four-sensor electrochemical cell was routinely operated at +50 mV, +100 mV, +450 mV and +800 mV. The Model 530 Fluorescence Detector was set for an excitation wavelength of 348 nm and an emission wavelength of 444 nm. The electrochemical guard cell was set at a potential of +800 mV to oxidize any contaminants in the mobile phase. The +450 mV channel provided the most sensitive response for measuring both BH₄ and XH₂ while the fluorescence detector, positioned just after the 4-channel electrochemical cell, was used for measuring B, P and BH₂.

Aerobic autoxidation and superoxide-mediated oxidation of BH₄

BH₄ (100 μM) in 0.1 M sodium phosphate buffer pH 7.4 containing 0.4 mM DTPA was incubated at 25°C in air. After 15, 30, and 60 min of incubation the reaction was stopped by adding ascorbic acid to a final concentration of 1 mg/ml and by acidification to pH 3 with phosphoric acid, ortho 85%. The main pteridines, BH₄, BH₂, B, P and XH₂ (Fig. 2) derived from BH₄ autoxidation were separated by HPLC and measured with electrochemical (BH₄ and XH₂) and fluorescence (B, P, BH₂) detectors.

Oxidation of 100 μM BH₄ in 0.1 M sodium phosphate buffer pH 7.4 containing 0.4 mM DTPA was performed in the presence of KO₂. Serial aliquots of KO₂ in DMSO anhydrous solution, corresponding to 'O₂⁻-released at final concentrations of 100, 50, and 25 μM, as calculated from the reduction of ferricytochrome *c* (50 μM) using an extinction coefficient of 21 mM⁻¹cm⁻¹ at 550 nm [31], were added to BH₄ solutions. After 5 min, SOD (1,000 U/ml), CAT (200 U/ml), ascorbic acid (1 mg/ml), and H₃PO₄ 85% (0.85%) were added to the medium. A blank in which SOD (20,000 U/ml) and CAT (200 U/ml) were present together with KO₂ was used. The reaction medium was loaded into Centricon filters (5,000 M.W. cutoff) and centrifuged for 60 min at 10,000 × g at 4°C. The filtrate was then injected into the HPLC column and analysed using electrochemical and fluorescence detectors.

BH₄ oxidation by hydrogen peroxide

To measure BH₄ oxidation by hydrogen peroxide (H₂O₂), BH₄ (100 μM final concentration) was dissolved in 0.1 M sodium phosphate buffer pH 7.4 containing 0.4 mM DTPA and treated with H₂O₂ at 25° C in air for 15 min. The oxidative effect was studied with an increasing concentration of H₂O₂ (0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 mM). To stop the BH₄ oxidation, ascorbic acid (1mg/ml), 200 U/ml CAT, and phosphoric acid, ortho 85% were added to the medium. Also, samples in which CAT was added before the addition of 4 mM H₂O₂, were evaluated. The reaction medium was treated as described above.

BH₄ oxidation by hydroxyl radical generated via the Fenton reaction

The hydroxyl radical generation from the Fenton reaction was performed using the iron chelate Fe(III) NTA as previously described [32] which occurs via the 2 step reaction below:

This Fe(III) chelate was prepared from addition of ferrous sulfate to nitrilotriacetic acid (nta) at a ratio of 1:2 in air at pH 5.5. Since BH₄ is unstable in air over prolonged time, these reactions measuring the time course of BH₄ oxidation were performed under anaerobic conditions under argon gas and initiated by adding H₂O₂ (0.4 mM) to 10 mM sodium phosphate buffer, pH 7.4, containing BH₄ (100 μM) and 0.1 mM Fe(III)-NTA₂.

After an incubation at 25°C for 15, 30, and 60 min, the reactions were stopped by adding CAT (200 U/ml), DTPA (0.4 mM), and ascorbic acid (1 mg/ml).

Measurement of hydroxyl radical generated by the Fenton reaction

The Fenton reaction was carried out in the presence of 5.0 mM D-Phenylalanine (D-Phe) as described previously [33]. The yield of hydroxylation products was obtained by HPLC separation as described by Biondi et al [34].

Statistical analysis

Data are presented as mean ± S.E.M., unless otherwise specified. Means for each of the measured variables were by one-way ANOVA and differences were considered significant at a P level of <0.05. Linear correlation was performed with use of GraphPad Prism 4.

RESULTS

Chromatographic analysis of pteridines

We utilized a method for the separation and direct detection of BH₄, BH₂, biopterin and pterin (B,P) and XH₂ by an isocratic HPLC method that was a modification of the Hyland method [27]. Figure 3 shows a representative fluorescence and electrochemical chromatogram of an injected standard mixture of pteridine species. Biopterin and pterin (B and P) are naturally fluorescent, whereas BH₂ has been converted to the fluorescent form by post-column electrochemical oxidation with the analytical electrode set to +800 mV [27]. BH₄ and XH₂ are not converted to a fluorescent species following electrochemical oxidation, therefore these were detected using an analytical electrode set to +450 mV. To validate this HPLC analysis for the determination of the pteridines, limit of detection (LOD) was evaluated. With regard to the limit of detection (LOD), XH₂, B, P, BH₂ and BH₄ values were 120, 80, 160, 100 and 60 fmol, respectively. High reproducibility of chromatographic separation was confirmed by a reproducibility of retention times (CV = 0.52 ± 0.04 %, recorded for all pteridines) and peak areas (CV = 1.71 ± 0.8 %, recorded for all pteridines) determined on seven standard mixtures measured on seven consecutive days.

BH₄ autoxidation

Figure 4 (left) shows a series of chromatograms of BH₄ oxidation in air as a function of incubation time. A marked trend of BH₄ oxidation by oxygen was detected with a decrease that was proportional to the incubation time. This was detected as a decrease in the peak area. The observed decrease in BH₄ was accompanied by the appearance of a peak corresponding to BH₂ detected as early as 15 min after the initiation, and its rise was proportional to the incubation time. B and P were detected as minor products. The quantitative time course of BH₄ air oxidation is depicted as histograms in Figure 4 (A–D, right), where the significant changes observed for BH₄ and for BH₂ are evident. The BH₄ level dropped by 14.5 ± 1.9 % after 15 min of incubation (Fig. 4B), and the trend continued after 30 min of incubation (55.3 ± 1.0 % decrease, Fig. 4C) reaching the final value of 11.5

$\pm 2.1 \mu\text{M}$ at the end of incubation (60 min, Fig. 4D). An opposite trend was noted for BH_2 with an increase after 15 min of incubation of $+7.0 \pm 0.5 \%$, and up to $+45.3 \pm 1.3 \%$ after 30 min; reaching a final value of $67.6 \pm 4.5 \mu\text{M}$ at the end of incubation. A significant correlation was detected between these two pteridines with $r^2 = 0.9606$. The other pteridines (B, P and XH_2) were slightly increased during the air incubation time with values ranging between 3.3 – 5.5 %. We examined the inhibitory effect of SOD toward BH_4 autoxidation using the incubation time of 60 min. In order to quench $\cdot\text{O}_2^-$ and H_2O_2 , we used SOD (1,000 U/ml) and CAT (200 U/ml) and we observed an inhibition of BH_4 autoxidation by a factor of five versus samples not incubated with SOD/CAT (data not shown). The overall sample recovery was in the range of 95%–98% for five experiments.

BH_4 oxidation by superoxide

Figure 5 shows the process of BH_4 oxidation as a function of the amount of KO_2^- released $\cdot\text{O}_2^-$. After treatment with $25 \mu\text{M}$ $\cdot\text{O}_2^-$ the BH_4 level ($73.3 \pm 3.6 \mu\text{M}$) decreased significantly ($P = 0.005$) with respect to the “ $0 \mu\text{M}$ $\cdot\text{O}_2^-$ ” control (sample in which BH_4 undergoes 5 min of autoxidation) value of $95.6 \pm 2.3 \mu\text{M}$ (a decrease of approximately 24%). This trend continued after $50 \mu\text{M}$ $\cdot\text{O}_2^-$ addition ($52.6 \pm 2.4 \mu\text{M}$) and decreased to a concentration of $19.5 \pm 2.5 \mu\text{M}$ at $100 \mu\text{M}$ $\cdot\text{O}_2^-$ treatment (a decrease of approximately 45% and 80%, respectively). As BH_4 decreased, a concomitant increase in BH_2 was observed. In fact, the BH_2 concentration increased to 22.6 ± 2.1 after addition of $25 \mu\text{M}$ $\cdot\text{O}_2^-$ and 42.5 ± 1.5 after addition of $50 \mu\text{M}$ $\cdot\text{O}_2^-$ reaching a value of $73.8 \pm 2.6 \mu\text{M}$ at the $100 \mu\text{M}$ $\cdot\text{O}_2^-$ concentration (an increase of approximately 77%). The other pteridines (B and P) did not change significantly with respect to control after $\cdot\text{O}_2^-$ treatment. The overall sample recovery was in the 94%–97% range for five experiments.

BH_4 oxidation by hydrogen peroxide

Figure 6A shows the HPLC chromatograms of the BH_4 oxidation products resulting from H_2O_2 treatment for a 15 min period at 25°C as a function of H_2O_2 concentration. At “ 0 mM H_2O_2 ” a small BH_2 signal was observed as the result of BH_4 autoxidation over this time. A gradual decrease in BH_4 peak area was apparent with increasing H_2O_2 concentration, with an opposite trend in BH_2 and XH_2 peak area. The B and P peaks also increased but were minor products. Analyzing the quantitative conversion of BH_4 to B and P in response to the H_2O_2 oxidant strength, changes in concentration were clearly evident (Fig. 6B). In particular, the BH_4 level was seen to decrease significantly ($P < 0.001$) only at H_2O_2 concentrations of 1 mM and higher. At 1 mM H_2O_2 , little oxidation was seen with $95.4 \mu\text{M}$ BH_4 remaining; while at 16 mM H_2O_2 , the BH_4 level dropped to $24.8 \pm 1.8 \mu\text{M}$ (a decrease of approximately 74%). Concomitant with the fall in BH_4 , there was a significant increase in B and P levels with the increase in H_2O_2 concentration. The measured XH_2 at 1 mM H_2O_2 was $1.9 \pm 0.2 \mu\text{M}$. The appearance of XH_2 significantly increased, correlating well with increasing H_2O_2 concentration ($r^2 = 0.9948$). Figure 7A shows the time course of BH_4 oxidation by 4 mM H_2O_2 under anaerobic conditions (Argon). The observed BH_4 concentration dropped by $22.4 \pm 0.7 \mu\text{M}$ after 15 min of incubation and an additional drop of $19.5 \pm 2.5 \mu\text{M}$ was seen after 30 min. At the end of the 60 min incubation period, the concentrations of the other oxidation products were (in μM): 24.5 ± 1.5 for BH_2 , 9.8 ± 1.5 for B, 10.9 ± 1.1 for P, and 22.7 ± 1.5 for XH_2 . These observed increases in oxidation product concentrations were correlated with the incubation time with r^2 values of 0.955, 0.912, 0.991 and 0.912 for BH_2 , B, P and XH_2 , respectively. The specificity of the conversion of BH_4 to these other products due to H_2O_2 was verified by adding CAT to the reaction medium, where it caused a complete inhibition of the observed H_2O_2 – induced oxidation of BH_4 (Fig. 7B). The overall sample recovery was in the 92%–96% range for five experiments.

BH₄ oxidation by $\cdot$ OH produced from the Fenton Reaction

As demonstrated in Fig. 8, $\cdot$ OH produced from the Fenton reaction under anaerobic conditions transformed the BH₄ into biopterins and pterins. A rapid fall of the BH₄ peak area was seen with the parallel increase in area of the XH₂ peak. These changes were related to incubation time. Quantitative measurements (Fig. 9A) confirmed the prominent decrease of BH₄ to $21.5 \pm 0.7 \mu\text{M}$, a value almost five times less with respect to the starting concentration ($100.3 \pm 2.8 \mu\text{M}$), at 15 min of incubation. With increasing incubation time the decrease continued, dropping to a level of $3.4 \pm 0.5 \mu\text{M}$ after 30 min and after 60 min BH₄ was completely consumed. XH₂ showed the opposite trend, appearing after 15 min of incubation followed by a continued increase reaching levels of $30.0 \pm 1.6 \mu\text{M}$ at the end of the incubation. BH₂ was prominent after 15 min of incubation with a value of $50.4 \pm 2.7 \mu\text{M}$. However, after 30 min of incubation, BH₂ concentration was not markedly increased ($55.5 \pm 1.2 \mu\text{M}$), indicating that the rate of BH₂ appearance was not linear with respect to incubation time; the BH₂ level started to decrease to a value of $37.9 \pm 1.9 \mu\text{M}$ at the end of incubation (perhaps due to the complete consumption of BH₄ and slower conversion of BH₂ to XH₂). Both P and B appeared after 15 min of incubation ($15.3 \pm 2.2 \mu\text{M}$ for P and $8.1 \pm 1.3 \mu\text{M}$ for B), and the rate of appearance of these oxidation products was also not linear with respect to time. In fact the P level increased significantly after 30 min of incubation ($P=0.008$) and then the levels did not change during the rest of the incubation period. B showed a similar trend, but the incubation time at which it reached its maximum was 15 min with a value of $8.1 \pm 0.26 \mu\text{M}$ (Fig. 9A). The time course of $\cdot$ OH production over the course of the experiment presented in Fig. 8A was evaluated by D-Phe hydroxylation. The specificity of the $\cdot$ OH-driven oxidation of BH₄ was confirmed using the $\cdot$ OH scavenger, DMTU, that when added to the reaction medium before starting the $\cdot$ OH production at a concentration of 0.6 M, was able to completely inhibit the biopterin and pterin formation. The same results were obtained when another $\cdot$ OH scavenger was used (Trolox at 2.5 mM, see Fig. 9B). The overall sample recovery was in the 93%–97% range for five experiments.

DISCUSSION

Pteridine concentrations have typically been determined in the fully aromatic form (B) by HPLC using fluorescence via pre-column I₂ oxidation of the reduced forms (BH₄, BH₂). In this oxidation method, originally described by Fukushima and Nixon [28], the oxidation of BH₄ and BH₂ in acidic media resulted in a quantitative conversion to the fully oxidized biopterin. Conversely, the oxidation of BH₄ at strongly alkaline pH caused a side-chain cleavage while BH₂ remained oxidized to B. Thus, the net yield of B from acidic vs. alkaline oxidation was used to calculate the tetrahydro- form. The indirect nature of this assay for the determination of the original oxidation state of B is a potential pitfall of this technique. Hyland [27] described a direct estimation of the main pteridines (BH₄, BH₂, P, B and XH₂) using HPLC with sequential electrochemical and fluorimetric detection. BH₄ and XH₂ were detected by electrochemical oxidation, while BH₂ by fluorescence following post-column electrochemical oxidation; fully oxidized pteridines (P and B) were detected by their natural fluorescence. A mobile phase consisting of KH₂PO₄ 0.1 M, EDTA 0.05 mM, DTT 0.16 mM and OSA 0.05 mM pH 2.5 and a 250 × 4.6 mm Apex 5μm HPLC column with flow-rate of 1.0 ml/min was employed. Using 20% methanol as elution reagent and degassing the mobile phase to prevent BH₄ autoxidation, the minimum detection limits for all examined pteridines were in the range of 300–540 fmol. A chromatography run time of 30 min was necessary. This assay was recently employed by our group to determine the biopterin and pterin content in ischemic rat hearts [26]; however, this approach had some limitations in terms of BH₄ stability and detection limit of the pteridines. Accordingly, in the present study we sought to further improve BH₄ chemical stability during the chromatographic run as well as sensitivity of the assay. Several modifications were thus introduced in the method. In particular, we

chose a mobile phase consisting of 6.5 mM NaH₂PO₄, 6 mM citric acid, 1 mM OSA, pH 3.01, and substituted DTPA 2.5 mM for EDTA and 2% acetonitrile (ACN) for 20% methanol; DTT concentration was also increased to 1 mM. The higher concentrations of thiol (DTT) and chelating reagent (DTPA) decreased the autoxidation rate of BH₄ (0.001 μM/min) and also proved suitable in an extraction buffer for biological samples [34, 35]. ACN, used as eluting reagent, improved BH₂, P, and B detection limits. With these changes, the analyte detection limits reached levels between 60–120 fmol. Furthermore, use of an Atlantis dC18 reverse phase column 5 μM (4.6 × 100 mm) (Waters) with flow rate set at 1.3 ml/min reduced the chromatographic elution time to 10 min.

It is well known that BH₄ is prone to autoxidation in the presence of molecular oxygen (O₂). The nature of the products formed after the autoxidation of BH₄ depends on several factors, including pH, temperature, buffer type and concentration [36]. When oxidation in air for 30 min at 25°C was carried out, approximately one third of the BH₄ was converted to BH₂, using the Fukushima and Nixon method [28, 37]. When BH₄ aerobic oxidation was studied using UV/VIS spectral changes, BH₂ was considered the predominant product but its formation did not proceed directly. Indeed, BH₄ oxidation initially produces an intermediate compound, quinonoid 5,6-dihydrobiopterin (qBH₂) that, because of its instability, undergoes a rearrangement to dihydropterin (PH₂) [36]. By means of two specific assays (eliciting luminescence from lucigenin and ESR-spectrometric detection of the DEPMPO-OOH radical adduct), the kinetics of the autoxidation process were established. This reaction occurred between BH₄ and O₂ and caused a release of 'O₂⁻ that served as an initiation reaction for the further, rapid reaction of the 'O₂⁻ with BH₄; thereby, very likely establishing a chain reaction process involving the reduction of O₂ by the intermediary H₃B[•] radical. The final product, resulting from the BH₄ autoxidation process, was BH₂ [38–40]. Of note, for applications to biological samples, this autoxidation must be considered. Prophylactic measures to avoid BH₄ oxidation during sample handling and preparation are required. In preliminary experiments with heart tissue, cells and plasma, we have found that adding 1 mM ascorbic acid, 1 mM DTT and 0.1 mM DTPA to de-aerated samples with addition of anaerobic low-pH buffer (2.5–3.0) can prevent BH₄ autoxidation.

Our results using the direct measurement of pteridines by HPLC confirmed, in part, the previous studies of BH₄ autoxidation. We confirmed that the major product of BH₄ oxidation in air was BH₂ with a value of 46.3 ± 1.3% after 30 min of incubation but that autoxidation also produces small amounts of P, B and XH₂ with values of 5.5 ± 0.3%, 3.7 ± 0.2% and 3.3 ± 0.1%, respectively (see Figure 4, A–D). Using EPR spin trapping competition experiments with DEPMPO, it was reported that a chain reaction process involving a BH₄^{•+} radical is an intermediate product in the reaction between BH₄ and 'O₂⁻ generated with the xanthine/xanthine oxidase system [41]. The HPLC assay used here also showed that BH₂ was the stable product generated from 'O₂⁻-dependent autoxidation, as shown in Figure 5.

We also confirmed the 'O₂⁻ production during the BH₄ autoxidation process by means of the inhibitory effect of SOD on the air-induced BH₄ transformation. A reduction of BH₂ formation was observed, with its generation inhibited by 80.0 ± 0.2% with respect to the value obtained after 60 min incubation with no SOD present. Moreover, the direct exposure of 100 μM BH₄ to 'O₂⁻, released from KO₂ in aqueous solution, demonstrated that 'O₂⁻ plays a role as a modulator in BH₄ aerobic autoxidation. Indeed, we found that direct addition of 50 μM 'O₂⁻ caused formation of 42.5 ± 1.5 μM BH₂ (see Figure 5). The oxidative effect of 'O₂⁻, formed during the BH₄ autoxidation, may explain the extreme susceptibility of BH₄ to degrade to BH₂. Therefore, we can well understand the beneficial vascular effect of molecules such as ascorbic acid that, by preventing the 'O₂⁻ induced BH₄ oxidation, maintain the physiological level of BH₄ and so maintain an optimal NO

bioavailability thus preventing endothelial dysfunction in both *in vitro* and *in vivo* conditions [42, 43].

Using the direct method and selecting an incubation time of 15 min to minimize the effect of autooxidation, the H_2O_2 oxidative-dependent molecular pathway of BH_4 degradation has been clarified. H_2O_2 caused BH_4 oxidation to XH_2 as a final and stable product when the concentration was greater than 1 mM and the oxidant conversion was proportional to the concentration of H_2O_2 , as shown in Figure 6. BH_4 , despite its high sensitivity to air oxidation, was decomposed only with H_2O_2 concentrations of 1 mM and higher. Since the physiological concentration of H_2O_2 is in the micromolar range, H_2O_2 -mediated oxidation of BH_4 *in vivo* would be minimal. In fact, it has been demonstrated that while H_2O_2 , peroxynitrite (ONOO^-), O_2^- and $\text{OH}^\bullet$ all have inhibitory effects on NOS-related NO production, only O_2^- and ONOO^- inhibit at pathophysiological levels. Subsequent addition of BH_4 fully restored activity after O_2^- exposure, while BH_4 only partially restored the activity decrease induced by the other three oxidants [44]. Interestingly, in the skin depigmentation disorder, vitiligo, the concentration of H_2O_2 has been reported as high as the millimolar range in the epidermis accompanied by low catalase levels and high concentrations of both 6- and 7-biopterin [45, 46]. At this H_2O_2 concentration level, our experimental results predict that BH_4 would be oxidized first to BH_2 which would then be further oxidized to XH_2 with increasing H_2O_2 concentration. As shown in Figure 6B, after a 15 min incubation at 16 mM H_2O_2 , the initial BH_4 has been oxidized by approximately 75% mainly to BH_2 and XH_2 . In a related study, the oxidation of BH_2 by H_2O_2 was shown to generate XH_2 with a bimolecular rate constant, k , of $(2.7 \pm 0.2) \times 10^{-2} \text{ M}^{-1}\text{s}^{-1}$ at pH 7.0 and 37° C. XH_2 was shown to be oxidized to xanthopterin (X) by H_2O_2 at a rate two orders of magnitude slower than that for BH_2 to XH_2 , implying that it is possible that X might accumulate *in vivo*, albeit rather slowly [47].

Major cardiovascular risk factors including hypercholesterolemia, diabetes mellitus, chronic smoking as well as hypertension, increase the vascular production of ROS, through the increased activity of enzymes such as NADPH oxidase and xanthine oxidase [15, 17, 18, 20, 48, 49]. This oxidative stress may result in BH_4 oxidation with a subsequent decrease of vascular bioavailability of NO. Another reported function of BH_4 is that it acts as a ROS scavenger [50]. In fact, it has been shown that BH_4 is able to protect various types of cells, including vascular endothelial cells, against the toxic effects of ROS [50, 51]. Therefore, BH_4 appears to be not only a critical cofactor for NOS but also a non-specific protective factor for vascular endothelial cells against oxidative stress, when it is in a free unbound form. To examine this function, we performed an experiment in which BH_4 was exposed to $\text{OH}^\bullet$. This oxygen radical was chosen because, among all ROS, the $\text{OH}^\bullet$ is by far the most reactive and plays a relevant role in the pathogenesis of disease states such as ischemia [52] and ischemia-reperfusion [48, 53]. The $\text{OH}^\bullet$, produced by the Fenton reaction under anaerobic conditions, caused depletion of BH_4 with rapid conversion to BH_2 that was then apparently oxidized to XH_2 with side-chain cleavage at the C_6 position (see Figures 8 and 9A). Therefore, it seems that BH_2 can be an intermediate in the $\text{OH}^\bullet$ -generated end products, mainly XH_2 but also a small amount of B. As shown in Figure 9B, BH_4 was found to be a highly potent scavenger, exceeding the potency of the highly effective $\text{OH}^\bullet$ scavenger DMTU commonly used in cellular systems to protect against $\text{OH}^\bullet$ [54]. Substantial differences were found on BH_4 chemical oxidation due to O_2^- and $\text{OH}^\bullet$. The Fenton anaerobic reaction with $\text{OH}^\bullet$ formation caused BH_4 transformation to the irreversible forms, P and XH_2 , while the O_2^- reaction stopped at the reversible and partially oxidized form, BH_2 .

In conclusion, a highly sensitive HPLC analytic method is reported that permitted simultaneous direct measurement of BH_4 and its pteridine derivatives using sequential

electrochemical and fluorimetric detection. This assay was utilized to demonstrate that the formation of the primary two-electron oxidation product, BH₂, was predominant during BH₄ autoxidation. Direct participation of $\cdot\text{O}_2^-$, as demonstrated by the SOD inhibiting effect, during air-mediated BH₄ oxidation was shown. The irreversible metabolite, XH₂, was produced during $\cdot\text{OH}$ -driven BH₄ oxidation. This improved HPLC method, would be applicable to measure BH₄ and its metabolites including, BH₂, B, P and XH₂ as an aid to diagnose diseases and pathological states like hypercholesterolemia, diabetes mellitus, heart failure, atrial fibrillation, alterations with chronic smoking, as well as hypertension, in which an increase in vascular ROS production is observed [17, 19, 20, 22, 55, 56]. This method would also be useful to monitor the plasma BH₄ level of patients under oral BH₄ therapy aimed to improve endothelial dysfunction to reduce the progression of atherosclerosis or other vascular diseases [57]. Application to biological samples requires prophylactic measures to avoid BH₄ oxidation during sample handling and preparation.

Acknowledgments

The authors wish to thank Carmen Ghita, Marco Bettini, and Livio Bottiglieri M.D. for their excellent technical assistance.

References

1. Bonafe L, Thony B, Leimbacher W, Kierat L, Blau N. Clin Chem. 2001; 47:477–485. [PubMed: 11238300]
2. Thony B, Auerbach G, Blau N. Biochem J. 2000; 347(Pt 1):1–16. [PubMed: 10727395]
3. Watschinger K, Keller MA, Golderer G, Hermann M, Maglione M, Sarg B, Lindner HH, Hermetter A, Werner-Felmayer G, Konrat R, Hulo N, Werner ER. Proc Natl Acad Sci U S A. 2010; 107:13672–13677. [PubMed: 20643956]
4. Rokos H, Beazley WD, Schallreuter KU. Biochem Biophys Res Commun. 2002; 292:805–811. [PubMed: 11944885]
5. Vignoni M, Cabrerizo FM, Lorente C, Claparols C, Oliveros E, Thomas AH. Org Biomol Chem. 2010; 8:800–810. [PubMed: 20135037]
6. Tayeh MA, Marletta MA. J Biol Chem. 1989; 264:19654–19658. [PubMed: 2584186]
7. Xia Y, Tsai AL, Berka V, Zweier JL. J Biol Chem. 1998; 273:25804–25808. [PubMed: 9748253]
8. Wei CC, Crane BR, Stuehr DJ. Chem Rev. 2003; 103:2365–2383. [PubMed: 12797834]
9. Wever RM, Luscher TF, Cosentino F, Rabelink TJ. Circulation. 1998; 97:108–112. [PubMed: 9443438]
10. Suwaidi JA, Hamasaki S, Higano ST, Nishimura RA, Holmes DR Jr, Lerman A. Circulation. 2000; 101:948–954. [PubMed: 10704159]
11. Stroes E, Kastelein J, Cosentino F, Erkelens W, Wever R, Koomans H, Luscher T, Rabelink T. J Clin Invest. 1997; 99:41–46. [PubMed: 9011574]
12. Scott-Burden T. Circulation. 1995; 91:248–250. [PubMed: 7805211]
13. Poredos P. Pathophysiol Haemost Thromb. 2002; 32:274–277. [PubMed: 13679656]
14. Cosentino F, Patton S, d'Uscio LV, Werner ER, Werner-Felmayer G, Moreau P, Malinski T, Luscher TF. J Clin Invest. 1998; 101:1530–1537. [PubMed: 9525996]
15. Landmesser U, Dikalov S, Price SR, McCann L, Fukui T, Holland SM, Mitch WE, Harrison DG. J Clin Invest. 2003; 111:1201–1209. [PubMed: 12697739]
16. Harrison DG. J Clin Invest. 1997; 100:2153–2157. [PubMed: 9410891]
17. Landmesser U, Harrison DG. Circulation. 2001; 104:2638–2640. [PubMed: 11723010]
18. Sorescu D, Weiss D, Lassegue B, Clempus RE, Szocs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor WR, Griendling KK. Circulation. 2002; 105:1429–1435. [PubMed: 11914250]
19. Laursen JB, Somers M, Kurz S, McCann L, Warnholtz A, Freeman BA, Tarpey M, Fukui T, Harrison DG. Circulation. 2001; 103:1282–1288. [PubMed: 11238274]

20. Vasquez-Vivar J, Duquaine D, Whitsett J, Kalyanaraman B, Rajagopalan S. *Arterioscler Thromb Vasc Biol.* 2002; 22:1655–1661. [PubMed: 12377745]
21. d'Uscio LV, Milstien S, Richardson D, Smith L, Katusic ZS. *Circ Res.* 2003; 92:88–95. [PubMed: 12522125]
22. Tiefenbacher CP, Bleeke T, Vahl C, Amann K, Vogt A, Kubler W. *Circulation.* 2000; 102:2172–2179. [PubMed: 11056088]
23. Cardounel AJ, Xia Y, Zweier JL. *J Biol Chem.* 2005; 280:7540–7549. [PubMed: 15574418]
24. Meininger CJ, Marinos RS, Hatakeyama K, Martinez-Zaguilan R, Rojas JD, Kelly KA, Wu G. *Biochem J.* 2000; 349:353–356. [PubMed: 10861247]
25. Shinozaki K, Kashiwagi A, Nishio Y, Okamura T, Yoshida Y, Masada M, Toda N, Kikkawa R. *Diabetes.* 1999; 48:2437–2445. [PubMed: 10580434]
26. Dumitrescu C, Biondi R, Xia Y, Cardounel AJ, Druhan LJ, Ambrosio G, Zweier JL. *Proc Natl Acad Sci U S A.* 2007; 104:15081–15086. [PubMed: 17848522]
27. Hyland K. *J Chromatogr.* 1985; 343:35–41. [PubMed: 4066860]
28. Fukushima T, Nixon JC. *Anal Biochem.* 1980; 102:176–188. [PubMed: 7356152]
29. Dionex. Technical Note. 2000; 12:1–8.
30. Ross MA. *J Chromatogr B Biomed Appl.* 1994; 657:197–200. [PubMed: 7952068]
31. Fridovich I. *J Biol Chem.* 1970; 245:4053–4057. [PubMed: 5496991]
32. Josephson RA, Silverman HS, Lakatta EG, Stern MD, Zweier JL. *J Biol Chem.* 1991; 266:2354–2361. [PubMed: 1846625]
33. Maskos Z, Rush JD, Koppenol WH. *Arch Biochem Biophys.* 1992; 296:521–529. [PubMed: 1321588]
34. Biondi R, Xia Y, Rossi R, Paolocci N, Ambrosio G, Zweier JL. *Anal Biochem.* 2001; 290:138–145. [PubMed: 11180947]
35. Howells DW, Smith I, Hyland K. *J Chromatogr.* 1986; 381:285–294. [PubMed: 3760086]
36. Davis MD, Kaufman S, Milstien S. *Eur J Biochem.* 1988; 173:345–351. [PubMed: 3360013]
37. Armarego WL, Randles D, Taguchi H. *Eur J Biochem.* 1983; 135:393–403. [PubMed: 6617639]
38. Davis MD, Kaufman S, Milstien S. *Proc Natl Acad Sci U S A.* 1991; 88:385–389. [PubMed: 1988938]
39. Milstien S, Katusic Z. *Biochem Biophys Res Commun.* 1999; 263:681–684. [PubMed: 10512739]
40. Kirsch M, Korth HG, Stenert V, Sustmann R, de Groot H. *J Biol Chem.* 2003; 278:24481–24490. [PubMed: 12714605]
41. Vasquez-Vivar J, Whitsett J, Martasek P, Hogg N, Kalyanaraman B. *Free Radic Biol Med.* 2001; 31:975–985. [PubMed: 11595382]
42. Kuzkaya N, Weissmann N, Harrison DG, Dikalov S. *J Biol Chem.* 2003; 278:22546–22554. [PubMed: 12692136]
43. Heller R, Unbehaun A, Schellenberg B, Mayer B, Werner-Felmayer G, Werner ER. *J Biol Chem.* 2001; 276:40–47. [PubMed: 11022034]
44. Sun J, Druhan LJ, Zweier JL. *Arch Biochem Biophys.* 2008; 471:126–133. [PubMed: 18201545]
45. Schallreuter KU, Moore J, Wood JM, Beazley WD, Gaze DC, Tobin DJ, Marshall HS, Panske A, Panzig E, Hibberts NA. *J Invest Dermatol Symp Proc.* 1999; 4:91–96.
46. Schallreuter KU, Moore J, Wood JM, Beazley WD, Peters EM, Marles LK, Behrens-Williams SC, Dummer R, Blau N, Thony B. *J Invest Dermatol.* 2001; 116:167–174. [PubMed: 11168813]
47. Dantola LM, Schuler TM, Denofrio MP, Vignoni M, Capparelli AL, Lorente C, Thomas AH. *Tetrahedron.* 2008; 64:8692–8699.
48. Zweier JL. *J Biol Chem.* 1988; 263:1353–1357. [PubMed: 2826476]
49. Zweier JL, Kuppusamy P, Lutty GA. *Proc Natl Acad Sci U S A.* 1988; 85:4046–4050. [PubMed: 2836868]
50. Shimizu S, Hiroi T, Ishii M, Hagiwara T, Wajima T, Miyazaki A, Kiuchi Y. *Int J Biochem Cell Biol.* 2008; 40:755–765. [PubMed: 18054268]
51. Ishii M, Shimizu S, Momose K, Yamamoto T. *J Cardiovasc Pharmacol.* 1999; 33:295–300. [PubMed: 10028940]

52. Powell SR, Hall D. *Free Radic Biol Med.* 1990; 9:133–141. [PubMed: 2172099]
53. Zweier JL, Kuppusamy P, Williams R, Rayburn BK, Smith D, Weisfeldt ML, Flaherty JT. *J Biol Chem.* 1989; 264:18890–18895. [PubMed: 2553726]
54. Wasil M, Halliwell B, Grootveld M, Moorhouse CP, Hutchison DC, Baum H. *Biochem J.* 1987; 243:867–870. [PubMed: 2821995]
55. Nishijima Y, Sridhar A, Bonilla I, Velayutham M, Khan M, Terentyeva R, Li C, Kuppusamy P, Elton TS, Terentyev D, Gyorke S, Zweier JL, Cardounel AJ, Carnes CA. *Cardiovasc Res.* 2011; 91:71–79. [PubMed: 21460065]
56. Talukder MA, Johnson WM, Varadharaj S, Lian J, Kearns PN, El-Mahdy MA, Liu X, Zweier JL. *Am J Physiol Heart Circ Physiol.* 2011; 300:H388–396. [PubMed: 21057039]
57. Cosentino F, Hurlimann D, Delli Gatti C, Chenevard R, Blau N, Alp NJ, Channon KM, Eto M, Lerch P, Enseleit F, Ruschitzka F, Volpe M, Luscher TF, Noll G. *Heart.* 2008; 94:487–492. [PubMed: 17916662]

Highlights

- A sensitive direct HPLC method is adapted to measure BH₄ and its metabolites.
- The effect of oxidants on BH₄ is determined and resulting metabolites measured.
- The primary product of BH₄ autoxidation is BH₂.
- The primary product of superoxide-catalyzed oxidation of BH₄ is BH₂.
- Hydroxyl radical driven BH₄ oxidation results primarily in dihydroxanthopterin.

Fig. 1.

Instrument configuration for HPLC with electrochemical and fluorimetric detection.

Fig. 2.
Molecular structures of the detected pteridines.

Fig. 3. HPLC chromatograms of a mixture of 25 μM XH₂, 50 μM B, 50 μM P, 100 μM BH₂ and 25 μM BH₄. FLUO is the spectrofluorimeter detector channel with excitation and emission set at 348 and 444 nm, respectively; 450 mV and 800 mV electrochemical detector channels are also shown. The limits of detection (LOD) are 120, 80, 160, 100 and 60 fmol for XH₂, B, P, BH₂ and BH₄, respectively.

Fig. 4.

Time-dependent series of BH₄ autoxidation HPLC chromatograms (left); quantitative determination of pteridines, produced from BH₄ autoxidation (right). Columns are the means of five experiments and the S.E.M. Recovery in the range of 94.8–98.3% for the series.

A, B, C and **D** represent the incubation times of reaction in minutes at 25°C, corresponding to 0, 15, 30 and 60 min, respectively.

Fig. 5. Histograms representing the pteridine levels derived from BH_4 oxidation by graduated concentrations of KO_2 - released $\cdot\text{O}_2^-$. The mean of five experiments are shown with S.E.M.

Fig. 6.

A) Series of H_2O_2 concentration-dependent BH_4 oxidation HPLC chromatograms; BH_4 (100 μM) in 0.1 M sodium phosphate buffer pH 7.4 containing 0.4 mM DTPA reacted with H_2O_2 of 0, 2, 4, 8 and 16 mM for 15 min at 25°C. B) H_2O_2 concentration effect on BH_4 oxidation. Results are expressed as pteridine concentration (μM). The columns are the means of five experiments and the bars are S.E.M. Recovery 94.2–97.3%.

Fig. 7.

A) 4 mM H₂O₂ effect on BH₄ oxidation in relation to incubation time under anaerobic conditions (Argon); B) A control sample (labelled Control), in which BH₄ solution was incubated only in air, sample labelled H₂O₂ showing 4 mM H₂O₂-driven BH₄ oxidation, and sample H₂O₂ + CAT showing that the oxidation of BH₄ is due to the H₂O₂ with catalase inhibiting the oxidation. The columns are the means of three experiments and the bars are S.E.M. Recovery was 92–96 % in both experiments.

Fig. 8. Series of HPLC chromatograms depicting BH_4 oxidation induced by the Fenton reaction $\cdot\text{OH}$ generating system under anaerobic conditions at 15 min intervals over the course of one hour.

Fig. 9.

A) Effect of $\cdot\text{OH}$ on BH_4 oxidation in relation to incubation time., and the total $\cdot\text{OH}$ exposure. The total $\cdot\text{OH}$, evaluated by D-Phe hydroxylation as shown on the abscissa. B) DMTU 0.6 M and Trolox 2.5 mM inhibition effects on 60 min $\cdot\text{OH}$ driven - BH_4 oxidation; Control is the sample, in which BH_4 solution was incubated for 60 min under argon. Results are expressed as pteridine concentration (mM). The columns are the means of five experiments and the bars are S.E.M. Recovery was 93–97 %.