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ISOLATION AND CHARACTERIZATION OF QUINONOID DIHYDROPTERINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The 6*R*- and 6*S*-diastereoisomers of 1-*erythro*-5,6,7,8-tetrahydrobiopterin were subjected to oxidation by ferricyanide at pH 3.5. High-performance liquid chromatography (HPLC) systems are described for the rapid analysis of the oxidation products. Using HPLC, enzymatic and spectroscopic techniques, it was demonstrated that both tetrahydrobiopterins initially were oxidized to labile quinonoid dihydrobiopterin intermediates, which subsequently rearranged with a half-life of about 5 min to form the stable 7,8-dihydrobiopterin. The 6*R*- and 6*S*-quinonoid dihydrobiopterins had unique chromatographic properties, but similar UV spectra.

INTRODUCTION

The most well defined rôle of 1-*erythro*-5,6,7,8-tetrahydrobiopterin (BH4) is as a cofactor in the enzymatic hydroxylation of phenylalanine, tyrosine and tryptophan[1]. In these reactions one atom of dioxygen is used in the hydroxylation of the aromatic amino acid while the other is reduced to water. Electrons are donated by the tetrahydrobiopterin cofactor, and for every mol of tetrahydrobiopterin that is oxidized, 1 mol of hydroxylated product (tyrosine, DOPA and 5-hydroxytryptophan, respectively) is formed. The overall reaction

$$RH + BH4 + O_2 \rightarrow ROH + BH2 + H_2O \quad (1)$$

is valid for the hydroxylation of all three amino acids. Although there is broad agreement on the validity of this overall reaction, the mechanism of hydroxylation as well as the number of intermediate compounds and their chemical nature are still controversial.

In the pioneering studies by Kaufman[2,3] it was assumed that an unstable quinonoid (quinoid) dihydropterin is the immediate product of the reaction from which the tetrahydro form is regenerated by a NADH (NADPH)-linked quinonoid dihydropteridine oxidoreductase. In accordance with this proposal, the labile dihydrobiopterins described in the present study will be designated q-BH2. Although further indirect experimental evidence has been given in support of quinonoid forms

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of dihydropterins[4], other structures and redox states have been assigned for the intermediate(s) which can be observed as a transitory spectral change both in the enzymatic and the non-enzymatic oxidation of the cofactor[5–7].

Most studies on the elementary reactions in aromatic amino acid hydroxylation have been performed by utilizing a number of different unconjugated reduced pterin cofactors, notably 6-methyl- (6-MPH4) and 6,7-dimethyl-5,6,7,8-tetrahydropterin (6,7-DMPH4). Due to recent advances in the synthesis and isolation of the naturally occurring cofactor, considered to be 1-*erythro*-5,6,7,8-tetrahydrobiopterin, in its pure form, we have approached the problem of characterization of the immediate oxidation products of BH4 by a combination of chromatographic, spectroscopic and enzymatic methods.

EXPERIMENTAL

Materials

Biopterin was supplied by Regis Chemical (Morton Grove, IL, U.S.A.) and Calbiochem (San Diego, CA, U.S.A.). These products were shown to be similar by high-performance liquid chromatography (HPLC) (see below), both corresponding to the natural isomer of biopterin, *i.e.*, 6-1-*erythro*-(1′,2′-dihydroxypropyl)-2-amino-4-hydroxypteridine[8,9]. An authentic sample of a mixture of 6*R*- and 6*S*-1-*erythro*-tetrahydrobiopterin was a gift from Hoffman-La Roche (Basle, Switzerland).

Potassium ferricyanide and ferrocyanide, as well as palladium oxide, were the products of E. Merck (Darmstadt, G.F.R.). All other reagents used in this study were of analytical quality.

Sheep liver dihydropteridine reductase (E.C. 1.6.99.7) and bovine liver dihydrofolate reductase (E.C. 1.5.1.3) were purchased from Sigma (St. Louis, MO, U.S.A.).

Absorption and fluorescence spectroscopy

Absorption spectra and enzyme activities were measured on a Cary Model 219 spectrophotometer (Varian, Palo Alto, CA, U.S.A.).

Fluorescence measurements were carried out using a Model SFM 23 spectrofluorometer from Kontron (Zürich, Switzerland). Fluorescence excitation and emission spectra were monitored by an X-Y recorder (Model 7035, Hewlett-Packard). No corrections were made for photomultiplier response, or monochromator sensitivity of xenon arc-lamp emission. The fluorescence intensity was expressed on a linear scale. Biopterin was measured on the basis of its native fluorescence (λ_{ex} = 365 nm and λ_{em} = 450 nm).

HPLC of tetrahydrobiopterin and its oxidation products

Tetrahydrobiopterin and its oxidation products were determined by HPLC using a Constametric III pump and variable wavelength UV detector (Spectro-Monitor I) from Laboratory Data Control (Riviera Beach, FL, U.S.A.). A recording integrator from Hewlett-Packard (Model 3380 A) was used for measurement of either area or peak height. The chromatographic separations were achieved at 20°C on either (1) a microparticulate bonded strong cation exchanger (Partisil-10 SCX, prepacked from Whatman, Clifton, NJ, U.S.A.), or (2) a reversed-phase silica support

(Spherisorb C_{18}, 10-μm particles, pre-packed from Phase Separations, Queensferry, Great Britain). Both analytical columns were packed in 25 cm × 4.6 mm I.D. stainless-steel tubes. A short precolumn (40 × 2 mm I.D. stainless-steel tube) packed with pellicular silica (HC Pellosil from Whatman) or pellicular reversed-phase silica support (C_{18}, 40-μm particles from Supelco, Bellefonte, PA, U.S.A.) was used for the two chromatographic systems.

In the cation exchange system, which was a modification of that published by Bailey and Ayling[10], the mobile phase (30 m*M* ammonium acetate, adjusted to pH 3.5 with acetic acid) was pumped at a flow-rate of 2.0 ml/min.

In the reversed-phase system, which was a modification of that published by Fukushima and Nixon[11], the mobile phase [10 m*M* potassium citrate, pH 3.5 containing 0.25% (v/v) 1-butanol] was pumped at a flow-rate of 3.0 ml/min.

A low pH value was selected for both chromatographic systems in order to reduce the rate of autoxidation of BH4, but pH < 3.5 was avoided due to the instability of the reversed-phase support at lower pH values. Furthermore, Matsuura *et al.*[12] have estimated the extinction coefficients of several tetrahydropterins at pH 3.5.

Each series of chromatographic runs was preceded by the injection of 10 μl of a freshly prepared 1 *M* solution of sodium dithionite as proposed by Bailey and Ayling[10]. Since metal ions accumulate on several HPLC supports[13] and pterins undergo several kinds of reactions with metal ions[14], the dithionite injections were routinely followed by injections of 20 μl of a 100 m*M* solution of EDTA.

Preparation of tetrahydrobiopterin diastereoisomers

Biopterin was reduced by hydrogen in 2.0 *M* acetic acid in water, using palladium oxide as the catalyst[10,12]. Acetic acid was selected due to its favourable properties in catalytic hydrogenation[15], also avoiding the deleterious effects of halides used in previous studies[10,12]. After 3 h of reduction, the mixture of 6*R*- and 6*S*-diastereoisomers thus obtained was applied to a Partisil SCX-M9 preparative HPLC column[12] to separate the two diastereoisomers. The solvent (deoxygenated 30 m*M* ammonium acetate, adjusted to pH 3.5 with acetic acid) was pumped at a flow-rate of 4.0 ml/min (3000 p.s.i.). The pure diastereoisomers recovered from the column were collected under nitrogen and stored in liquid nitrogen.

Using the same procedure, pure 7,8-dihydrobiopterin was isolated following partial hydrogenation of biopterin. The concentration of 7,8-BH2 was measured using the absorptivity of 6.2 (1 m*M*) at 330 nm and pH 6.8 (ref. 16).

The concentration of BH4 in stock solutions was determined spectrophotometrically at pH 1.0 and 3.5, using the extinction coefficients reported by Matsuura *et al.*[12] (see also Table I).

Enzyme assays

The dihydropteridine reductase and dihydrofolate reductase assays were performed at 25°C using modifications of previously published procedures[17,18]. The dihydropteridine reductase assay mixture contained 25 μ*M* NADH and 0.4 enzyme units per assay. The dihydrofolate reductase assay mixture contained 25 μ*M* NADPH and 0.1 enzyme units per assay. Both enzyme mixtures, in a final volume of 1.0 ml, contained 100 m*M* HCl–imidazole buffer (pH 6.8), and the reactions were initiated by

the addition of 10 nmol of 6*R*-BH4 oxidation products. In both instances, the reference cuvette contained a complete assay mixture, with water substituting for the enzymes. The reaction rates were calculated from the decrease in absorbance at 340 nm during the first 30 sec of the assay.

RESULTS

Oxidation of tetrahydrobiopterin by ferricyanide

The oxidation of the two 6-diastereoisomers of BH4 by ferricyanide at pH 3.5 was found to be complete in less than 10 sec, in complete agreement with the observations of Archer *et al.*[19] on the rates of oxidation of other tetrahydropterins. Complete oxidation of both diastereoisomers to the "immediate" products observed spectrally was obtained at a molar ratio of ferricyanide to BH4 of 2.0:1.0. In the various oxidation experiments to be described, we used a slight (5–25 %) excess of ferricyanide in order to guarantee the complete oxidation of the tetrahydrobiopterins. The application of such a small excess of oxidant did not result in any significant oxidation of dihydrobiopterins during the first 20 min of incubation (Fig. 5A).

Archer and Scrimgeour[20] described the spectral changes which occur during oxidation of 6,7-DMPH4 by a stoichiometric amount of ferricyanide at pH 8.0 under anaerobic conditions. From Fig. 1 it is seen that similar spectral changes are observed at pH 3.5 when the oxidation of the 6*R*- and 6*S*-diastereoisomers of BH4 by ferricyanide was followed under aerobic conditions. Using a slight excess of the oxidant

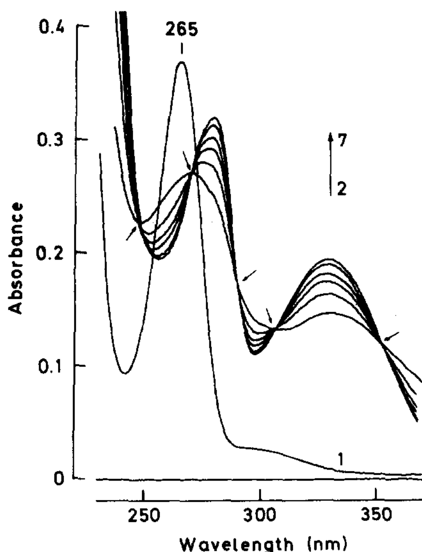

Fig 1. Oxidation of 6*R*.BH4 (λ_{max} = 265 nm) to q-BH2 (λ_{max} = 265 nm, see Fig. 6), and its subsequent rearrangement to 7,8-BH2 (λ_{max} = 278 nm). 224 nmol of ferricyanide were added to 104 nmol of 6*R*-BH4 in a final volume of 3.0 ml at 25°C. Simultaneously, 204 nmol of ferrocyanide plus 20 nmol of ferricyanide were added to the reference cuvette containing buffer only. 10 m*M* potassium citrate buffer, pH 3.50. Initial spectrum of 6*R*-BH4 (1) and spectra obtained 30, 50, 270, 390, 630 and 870 sec after the addition of ferricyanide (2–7).

TABLE I

ABSORPTIVITY OF TETRAHYDROBIOPTERIN DIASTEREOISOMERS AND THEIR DIHYDRO OXIDATION PRODUCTS

Compound	*6R-isomer*		*6S-isomer*	
	λ (nm)	*ε (mM⁻¹ cm⁻¹)*	*λ (nm)*	*ε (mM⁻¹ cm⁻¹)*
BH4*	265	12.2	266	12.6
BH2	248	6.9	248	6.9
(Isosbestic	270	8.2	669	7.9
points for the	290	5.8	290.5	5.5
q-BH2 → 7,8-BH2	305.5	3.6	307	4.1
transition)	352	4.2	347	4.5

* From Matsuura *et al.*[12].

(ferricyanide:BH4 = 2.2:1.0 on a molar basis) a rapid shift in the spectrum was observed, followed by a slower change (Fig. 1); the isosbestic points and extinction coefficients (at 1 m*M*) at these wavelengths are given in Table I. The shift in the position of the isosbestic points at pH 3.5 *versus* pH 6.8 (data not shown) is explained by the pronounced effect of pH on the UV spectra of pterins in general.

Characterization of the oxidation products of 6R-BH4 by HPLC

From Figs. 1 and 2 it is clear that an incubation mixture of 6*R*-BH4 and ferricyanide consists of at least three components with distinct spectral (Fig. 1) and

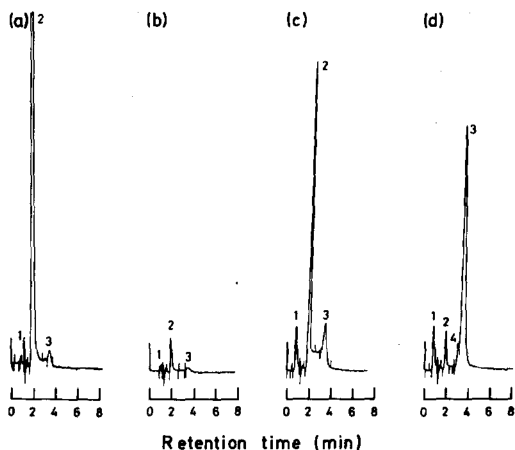

Fig. 2. Elution profile of 6*R*-BH4 and its oxidation products on C_{18} reversed-phase chromatography. a and b, 7.5 nmol of 98.7% pure 6*R*-BH4 before oxidation; detection at 265 nm (a) and 352 nm (b). c and d, 7.5 nmol of 6*R*-BH4 oxidation products analysed 30 sec (c) and 10 min (d) after the initiation of oxidation. 55 nmol of ferricyanide were added to 25 nmol of 6*R*-BH4 in a final volume of 100 μl and incubated at 25°C; 30-μl samples were injected at various time intervals during the oxidation cycle. The incubation medium contained 10 m*M* potassium citrate and 3 m*M* ammonium acetate, pH 3.50. Peaks: 1 = solvent front including ferricyanide–ferrocyanide (c and d); 2 = 6*R*-BH4 and 6*R*-q-BH2 with apparently identical retention times; 3 = 7,8-BH2; 4 = biopterin.

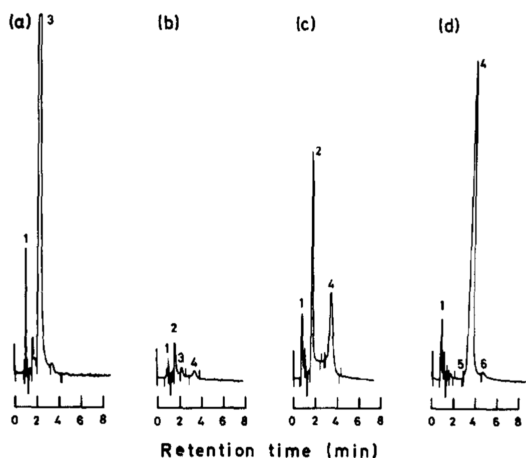

Fig. 3. Elution profile of 6*S*-BH4 and its oxidation products on C_{18} reversed-phase chromatography. The ferricyanide oxidation was performed as described in the legend to Fig. 2. a and b, 7.5 nmol of 99.0% pure 6*S*-BH4 before oxidation; detection at 266 nm (a) and 347 nm (b), c and d, Oxidation mixture injected 30 sec (c) and 10 min (d) after the initiation of oxidation; detection at 347 nm. Peaks: 1 = solvent front including ferricyanide–ferrocyanide (c and d); 2 = 6*S*-q-BH2; 3 = 6*S*-BH4; 4 = 7,8-BH2; 5 = biopterin; 6 = not identified.

chromatographic (Fig. 2) properties, and that the relative proportion of the components varies as a function of the incubation time. To circumvent these problems in HPLC detection, a wavelength corresponding to an isosbestic point of the 6*R*-q-BH2 → 7,8-BH2 transition, *i.e.*, $\lambda = 352$ nm was used. The molar absorptivity estimated at the selected isosbestic point (Table I) was used for quantitation of the individual components.

Using the same experimental conditions as in the spectroscopic studies (see legend to Fig. 1), 30-μl fractions of the incubation mixture were removed at selected time intervals and injected into the liquid chromatograph. In the reversed-phase

Fig. 4. Elution profile of 6*R*-q-BH2, 6*S*-q-BH2 and 7,8-BH2 on C_{18} reversed-phase chromatography. A mixture of 6*R*-BH4 (3.4 nmol) and 6*S*-BH4 (3.4 nmol) was oxidized by 21 nmol of ferricyanide and analyzed 30 sec after initiation of oxidation. Incubation conditions as described in Fig. 2. Peaks: 1 = solvent front including ferricyanide–ferrocyanide; 2 = 6*S*-q-BH2; 3 = 6*R*-q-BH2; 4 = 7,8-BH2.

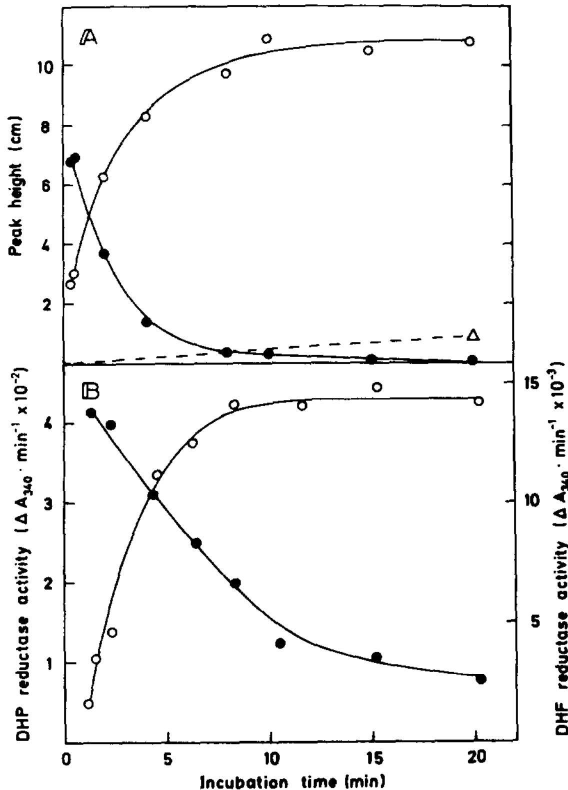

Fig. 5. A, Time-course of the disappearance of 6*R*-q-BH2 (●) and appearance of 7,8-BH2 (○) as measured from peak heights in C_{18} reversed-phase chromatography. The oxidation of 6*R*-BH4 by ferricyanide was initiated at zero time. Experimental conditions as in Fig. 2. A small amount of biopterin (△) was formed after prolonged incubation. This was confirmed by an identical HPLC analysis using fluorimetric detection. B, Time-course of the changes in cofactor activity of the oxidation mixture in the dihydropteridine (DHP) reductase (●) and dihydrofolate (DHF) reductase (○) assay systems. For details, see Experimental section.

system, detection at λ = 352 nm revealed only two chromatographic peaks during the first part of the oxidation cycle. The component with retention time (t_R) 1.85 min was identified as 6*R*-BH4 before the oxidant was added. Immediately after initiation of oxidation by ferricyanide an increase in both peak height and integrated peak area was observed for this component (Figs. 2 and 5A); thereafter it gradually decreased to a non-detectable level after *ca.* 20 min (Fig. 5A). The other component, with t_R = 3.35 min, was identified as 7,8-BH2 and found to increase progressively reaching an asymptotic level at about 15 min (Figs. 2 and 5A).

That the peak at t_R = 1.85 min actually represented two different components was shown by taking UV spectra of the peak before and after the addition of ferricyanide.

A typical spectrum of 6*R*-BH4 at pH 3.5 was observed before the addition of the oxidant (data not shown), whereas a spectrum characteristic of its immediate oxidation product was found for the peak appearing after oxidation (Fig. 6). The

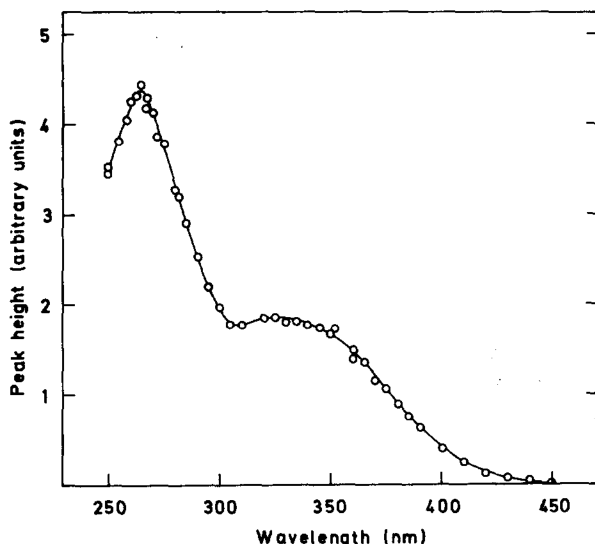

Fig. 6. UV spectrum of 6*R*-q-BH2 isolated by reversed-phase chromatography (see text). Identical oxidation mixtures prepared from 5.7 nmol of 6*R*-BH4 and 17.1 nmol of potassium ferricyanide in 10 n*M* potassium citrate, pH 3.5 were incubated for 40 sec at 25°C before injection into the liquid chromatograph. Each point represents separate assay mixtures analysed at different wavelengths.

spectrum of the second peak (t_R = 3.35 min) was found to be identical to that of authentic 7,8-BH2 (data not shown).

Further identification of the two oxidation products was obtained by measuring their ability to function as substrates in the dihydropteridine reductase and dihydrofolate reductase systems. As is seen from Fig. 5B, the component with t_R = 1.85 functioned as a substrate in the dihydropteridine reductase system, whereas that with t_R = 3.35 min was active in the dihydrofolate reductase system.

It should be noted that, upon prolonged incubation, a small peak of 1-*erythro*-biopterin was observed with t_R = 2.97 min.

The same series of experiments were also carried out using the HPLC system with a strong cation exchanger. The results obtained were in principle the same as described above, but due to the longer retention time of 6*R*-q-BH2 in this system (about 3 min) it was less suitable for demonstration of the early events during the oxidation process. However, the spectra obtained for the isolated intermediates were similar to those obtained using the reversed-phase HPLC system (data not shown).

Characterization of the oxidation products of 6S-BH4 by HPLC

For comparison, the 6*S*-diastereoisomer of tetrahydrobiopterin (6*S*-BH4) was subjected to the same series of oxidation experiments as 6*R*-BH4. The oxidation of 6*S*-BH4 by ferricyanide revealed similar kinetics and stoichiometry as 6*R*-BH4, and the spectral changes were almost identical. Distinct differences were, however, observed in the chromatographic properties of the two isomers (Fig. 3).

In the reversed-phase system detection at λ = 347 nm revealed three chromatographic peaks during the first part of the oxidation cycle. The component with t_R = 2.21 min was identified as 6*S*-BH4 before the oxidant was added. Immediately after

TABLE II

RETENTION TIMES FOR THE BIOPTERIN DERIVATIVES STUDIED

Conditions for the two chromatographic systems were as described in the text. Each value is an average from three to five assays. Standard deviations in parentheses.

Compound	*Chromatographic system*	
	C_{18}	*Partisil SCX*
Biopterin	2.97 (0.01)	1.79 (0.00)
7,8-BH2	3.35 (0.01)	1.97 (0.01)
6*R*-q-BH2	1.85 (0.00)	2.86* (0.00)
6*S*-q-BH2	1.65 (0.00)	2.94* (0.01)
6*R*-BH4	1.85 (0.01)	2.89* (0.00)
6*S*-BH4	2.21 (0.00)	3.38 (0.00)

* These three substances were not separated on this column.

initiation of oxidation by ferricyanide a peak was observed with t_R = 1.65 min. This component gradually decreased to a non-detectable level after *ca.* 20 min similar to that described in the experiments with the 6*R*-diastereoisomer (Fig. 5A). The third component, with t_R = 3.35 min, was identified as 7,8-BH2 and found to increase progressively reaching an asymptotic level at about 20 min.

Thus, the two 6-diastereoisomers of BH4 were initially oxidized to two chromatographically distinct, but spectrally very similar labile dihydrobiopterins (6*R*-q-BH2 and 6*S*-q-BH2), both of which subsequently rearranged to form the stable 7,8-BH2 (Fig. 4).

The chromatographic properties of 6*R*- and 6*S*-BH4 and their respective oxidation products are given in Table II.

DISCUSSION

HPLC with UV, fluorescence or electrochemical detection has recently been introduced for the rapid and sensitive assay of tetrahydropterins and their stable oxidation products (pterins and 7,8-dihydropterins) formed as a result of autoxidation[21] or other non-enzymatic oxidations[22]. These techniques have increasingly been applied to the analysis of pterins from a diversity of biological materials. So far, no attention has been given to the chromatographic properties of the quinonoid dihydropterins in the different HPLC systems developed for their related compounds, although they are considered to be the key intermediates during tetrahydropterin-dependent hydroxylations of aromatic amino acids[3].

In the present study the chromatographic conditions have been optimized for the detection and assay of quinonoid dihydrobiopterins (q-BH2). Reversed-phase chromatography was selected due to the short retention time of q-BH2 (t_R = 1.85 min for the 6*R*-isomer and t_R = 1.65 min for the 6*S*-isomer), partly eliminating the problem of its conversion to 7,8-BH2 during the chromatographic separation. In addition, the separation of the two 6-diastereoisomers of q-BH2 was found to be superior in the reversed-phase system (Table II). In all HPLC systems tested, however, we found that 6*R*-BH4 and its q-BH2 product had very similar chromatograph-

ic properties (Table II). A clear distinction between the two components could, however, be made by appropriate selection of the wavelength of detection, *i.e.*, λ = 265 nm for 6*R*-BH4 and λ = 352 nm for 6*R*-q-BH2 (Fig. 1). In the case of 6*S*-isomers, q-BH2 and BH4 were well separated with the retention times of 1.65 min and 2.21 min, respectively. It should be mentioned, however, that some conversion of q-BH2 into 7,8-BH2 occurred on the column for both diastereoisomers, which is evident from the lack of baseline separation of the two components (Figs. 2c and 3c) which differed by 1.50 min in their retention times (for the *R*-form). This fact has to be considered in the calculation of product yields.

Using conventional spectroscopy we observed that 6*R*-BH4 is rapidly oxidized by ferricyanide at pH 3.5, and the first spectrum recorded (started at 30 sec following addition of the oxidant) is already a mixed spectrum of q-BH2 (main component) and some 7,8-BH2. The proportion of 7,8-BH2 increases with time (Figs. 2 and 5) and the isosbestic points in the spectral transition (Table I) were selected as reference wavelengths for HPLC detection. Notably, λ = 352 nm (for the 6*R*-form) was very useful since BH4 was almost devoid of absorbance at this wavelength. Using the common extinction coefficient of 6*R*-q-BH2 and 7,8-BH2 at λ = 352 nm, the amount of the two oxidation products was estimated by HPLC using chromatographically pure 7,8-BH2 as a reference. From Fig. 5 it is seen that the disappearance of 6*R*-q-BH2 (Fig. 5A) is correlated in time to the reduction in substrate activity as determined by the quinonoid dihydropteridine reductase assay and the increase in substrate activity of the dihydrofolate reductase assay (Fig. 5B). The observed time-courses strongly support the identification of 7,8-BH2, and furthermore that the 6*R*-q-BH2 observed is actually the substrate for dihydropteridine reductase as proposed[3]. It is notable that the decrease in activity in the dihydropteridine reductase assay occurred more slowly than the increase of dihydrofolate reductase activity. One possible explanation for this is that the dihydropteridine reductase was slightly contaminated by dihydrofolate reductase.

Due to the lability of q-BH2, no true spectrum has so far been presented for these compounds[3] (see also Fig. 1). However, based on HPLC, the spectrum of a chromatographically pure q-BH2 was obtained both for the 6*R*- and the 6*S*-isomers. The absorption spectrum of the 6*R*-isomer consists of a strong band in the far UV region (at around 265 nm) and a weaker band centered around 320–325 nm, extending as far as 450 nm. This spectrum supports the early proposal of Kaufman[3] of a quinonoid structure for this labile intermediate.

The labile intermediates (6*R*-q-BH2 and 6*S*-q-BH2) formed by chemical oxidation of 6*R*-BH4 and 6*S*-BH4 were shown to be chromatographically distinguishable (Fig. 4), but spectrally very similar. This indicates that the stereochemical configuration at position 6 of the pteridine ring is preserved during the early stage of oxidation of BH4 and that the terminology 6*R*-q-BH2 and 6*S*-q-BH2 is justified, in agreement with the structures proposed by Kaufman[3]. Furthermore, Armarego[23] demonstrated a slight difference in dihydropteridine reductase activity with the H_2O_2 oxidation products of 6*R*- and 6*S*-6MPH4 using the human liver enzyme, also favouring the preservation of stereochemical configuration at the 6 position during oxidation.

The fact that the labile intermediate 6*R*-q-BH2 has a retention time indistinguishable from that of 6*R*-BH4 implies that one has to be very careful when measuring 6*R*-BH4 by means of reversed-phase or strong cation exchange HPLC.

This problem has not been considered in previous HPLC assays of tetrahydrobiopterins[10,12]. The 6*R*-BH4 peak (Fig. 2a) may well contain significant amounts of the quinonoid dihydrobiopterin, arising from the autoxidation of 6*R*-BH4. The contribution of 6*R*-q-BH2 can, however, easily be measured by using a wavelength setting at which the absorption of tetrahydropterins is minimal (Figs. 1 and 2).

Based on evidence obtained from the oxidation of various tetrahydropterins, Hemmerich[4] and Kaufman[2,3] suggested that the primary oxidation products of tetrahydropterins were quinonoid dihydropterins. These substances were postulated to be general intermediates, occurring as a result of chemical as well as enzymatic oxidation of tetrahydropterins. Based on spectral characteristics, Kaufman[3] favoured the *para*-quinonoid isomer in the case of DMPH4 oxidation. On the other hand, Wahlefeld *et al.*[5] presented evidence supporting the existence of a relatively stable trihydropterin radical during anaerobic ferricyanide oxidation of the 6,8-variant of DMPH4. Several related radicals have been characterized by EPR spectroscopy[24]. Later, various 4a (or 8a) adducts of oxygen and tetrahydropterins were reported as the primary products of enzymatic[6,25], as well as non-enzymatic oxidation of tetrahydropterins[7]. These oxygen-activated species may be the precursors of C_{4a}–N_5 bond-cleaved intermediates, which are postulated to occur during the phenylalanine-4-monooxygenase-catalyzed tetrahydropterin oxidation[26]. Recently, Hénichart *et al.*[27], using ultraviolet, nuclear magnetic resonance and mass spectroscopy, were unable to obtain evidence supporting the existence of quinonoid dihydropterins during peroxidation (H_2O_2-peroxidase) or autoxidation of DMPH4. Instead, they attributed the spectral changes observed to various oxygen-activated pterins[27].

The present work confirms the existence of well defined, labile dihydrobiopterins, occurring as obligate intermediates during ferricyanide oxidation of 6*R*- and 6*S*-BH4. Using conventional HPLC and UV spectroscopy as analytical tools, these were the only intermediates observed and the UV spectra are consistent with those of quinonoid dihydrobiopterins. However, the exact chemical structures of the quinonoid dihydropterins remain to be settled. These observations are not in conflict with the hypothesis that tetrahydropterin oxidation occurs by two one-electron steps. The primary products of this reaction are probably too short-lived to be detectable by the present approach.

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