Journal of Chromatography, 494 (1989) 77–85
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4882

DETERMINATION OF QUINONOID DIHYDROBIOPTERIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROCHEMICAL DETECTION

SIMON HEALES and KEITH HYLAND*

Department of Child Health, Institute of Child Health, 30 Guilford Street, London WC1N 1EH (U K)

(First received October 19th, 1988, revised manuscript received May 27th, 1989)

SUMMARY

Sodium bisulphite is shown to react with quinonoid dihydrobiopterin to form a stable adduct Sodium bisulphite does not react with tetrahydrobiopterin Quinonoid dihydrobiopterin reacts with dithioerythritol to form tetrahydrobiopterin, whereas the quinonoid dihydrobiopterin bisulphite adduct does not Using these properties we have developed an indirect method for the quantitative measurement of quinonoid dihydrobiopterin The method requires division of a sample into two Dithioerythritol is added to one half (*a*) This converts quinonoid dihydrobiopterin to tetrahydrobiopterin and prevents the oxidation of tetrahydrobiopterin Measurement of the tetrahydrobiopterin content of this sample by electrochemistry following high-performance liquid chromatographic separation (with dithioerythritol present in the mobile phase to prevent autoxidation of the tetrahydrobiopterin on column), therefore provides a total value of the tetrahydrobiopterin plus quinonoid dihydrobiopterin present within the original sample Sodium bisulphite is added to the other portion of the sample (*b*), followed immediately by dithioerythritol which prevents autoxidation of the remaining tetrahydrobiopterin The bisulphite reacts with the quinonoid dihydrobiopterin present and the quinonoid dihydrobiopterin–bisulphite adduct is no longer detected by electrochemistry at the retention time of tetrahydrobiopterin Using reversed-phase high-performance liquid chromatography and redox electrochemical detection, measurement of tetrahydrobiopterin in the absence (*a*) and presence (*b*) of bisulphite enables the concentration of quinonoid dihydrobiopterin to be calculated by subtraction ($a-b$) This method is shown to be quantitative and preliminary experiments demonstrate that it can be adapted for biological samples

0378-4347/89/$03 50

INTRODUCTION

Tetrahydrobiopterin (BH4) is the cofactor required for the enzymatic hydroxylation of phenylalanine, tyrosine and tryptophan [1] In these reactions BH4 is oxidized to quinonoid dihydrobiopterin (qBH2) which is then reduced back to BH4 by the pyridine-dependent enzyme, dihydropteridine reductase (DHPR) (EC 1 6 99 7) [2]. Failure of this salvage pathway may therefore result in accumulation of qBH2. In vitro, qBH2 has been shown to be very unstable, rapidly rearranging to form 7,8-dihydrobiopterin (BH2) and 7,8-dihydropterin (PH2) at neutral pH and above, and BH4 and biopterin (B), via a disproportionation reaction, at pH 3–4 [3].

A number of methods have been described for the detection of qBH2 using high-performance liquid chromatography (HPLC) with either ultraviolet (UV) or electrochemical detection (ED) [4,5]. Using reversed-phase HPLC systems it has proven difficult to separate qBH2 from BH4, however, it has been shown that qBH2 can be distinguished from BH4 electrochemically by using dual parallel adjacent amperometric detection in which one electrode is set at a negative potential for the detection of qBH2 and the other at a positive potential for the measurement of BH4 [6]. Although this methodology can be used to detect qBH2 and BH4 it is not suitable for the accurate measurement of these pterins in biological samples, since BH4 is readily oxidised via qBH2, and qBH2 itself rapidly undergoes tautomerisation and disproportionation reactions [3,7,8]. Consequently, considerable breakdown may occur during sample handling or during separation on the analytical column unless adequate steps are taken to prevent this occurring

In the method we have previously described for BH4 measurement, the addition of dithioerythritol (DTE) to samples and to the analytical mobile phase prevents BH4 oxidation [8], both during sample handling and during separation on the analytical column However, in the presence of thiol-containing antioxidants, any qBH2 present is reduced to BH4 [9,10]. This system therefore measures the total BH4 and qBH2 content of a sample as a single BH4 peak and is not suitable for the direct analysis of qBH2.

Recently, sodium bisulphite has been shown to form a stable adduct with qBH2 (Fig. 1) [3,7]. In this report we describe how formation of this adduct

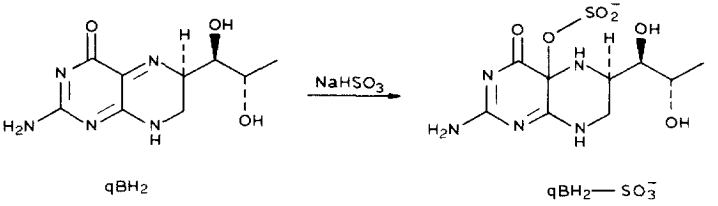

Fig 1 Reaction of qBH2 with sodium bisulphite ($NaHSO_3$)

can be used to indirectly measure the qBH2 concentration in chemically defined and biological samples.

EXPERIMENTAL

Chemicals

5,6,7,8-Tetrahydrobiopterin dihydrochloride, 5,6,7,8-tetrahydropterin dihydrochloride, 7,8-dihydrobiopterin and biopterin were supplied by Dr. B Schirks (Jona, Switzerland). 7,8-Dihydropterin was produced by dichlorophenolindolphenol oxidation of 5,6,7,8-tetrahydropterin [11] Sodium bisulphite and DTE were obtained from Aldrich (Gillingham, U K.). Horseradish peroxidase and dichlorophenolindolphenol were purchased from Sigma (Poole, U K). Potassium ferricyanide, hydrogen peroxide and ethylenediaminetetraacetic acid (EDTA) were obtained from BDH (Poole, U K).

Apparatus

HPLC-ED of BH4 This was performed essentially as previously described [8]. The chromatographic system consisted of a Spectra-Physics SP8770 pump (San Jose, CA, U.S.A.), a Rheodyne 7125 injector (Cotati, CA, U.S A.), a 5-μm ODS guard column (1 cm$\times$0 3 cm I D.) (HPLC Technologies, Macclesfield, U.K.) and a 5-μm Apex ODS (25 cm$\times$4.5 cm I D) column (Jones Chromatography, Llanbradach, U.K.). Detection was by an ESA Coulochem 5100A electrochemical detector using a Model 5011 high-sensitivity electrode (Bedford, MA, U S.A.) with upstream and downstream electrodes set at +0.06 and −0.46 V, respectively. Signals from the downstream electrode were detected by a Spectra-Physics SP4270 computing integrator.

HPLC-UV of BH4, qBH2, BH2, PH2 and B The chromatographic system was as above, except that detection was by a Pye Unicam LC 871 UV–visible detector at 220 nm

Mobile phases

Electrochemical detection The mobile phase consisted of sodium acetate (50 mmol/l), citric acid (5 mmol/l) pH 5 2, containing EDTA (48 μmol/l) and DTE (0 6 mmol/l) The mobile phase was degassed by sonication. The flow-rate was 1.3 ml/min and the column temperature was maintained at 40°C.

UV detection of BH4, qBH2, BH2 and B As above except the DTE and EDTA were omitted.

Generation of qBH2 qBH2 was formed from BH4 by the addition of potassium ferricyanide (ferricyanide/BH4 molar ratio 2 1). Formation of qBH2 under these conditions has been reported to be complete in a few seconds [6]. qBH2 was also generated at pH 7.4 by peroxidase (0.8 μg/ml) and hydrogen peroxide (17 μmol/l) [2].

UV spectroscopy of qBH2 and qBH2-SO_3 UV spectra were obtained using a Perkin-Elmer Lambda 5 dual-beam recording spectrophotometer

RESULTS AND DISCUSSION

The method for BH4 measurement used in the present study is the same as that previously described by us [8]. This method uses in series dual-electrode redox coulometric ED following reversed-phase HPLC [8] and involves oxidation of BH4 to qBH2 at the upstream electrode (E1) and subsequent reduction and measurement at the downstream electrode (E2) DTE is included in the mobile phase to prevent BH4 oxidation [8]. To demonstrate that DTE in the mobile phase could also convert qBH2 to BH4 [9,10], a qBH2 solution was generated by ferricyanide oxidation of BH4 and a sample immediately injected onto the HPLC column A single peak with a retention time identical to BH4 (Fig. 2a) was detected at E2 (Fig 2b) Furthermore, detection at E2 was only possible with prior oxidation at E1 (not shown), confirming that the compound measured was BH4 and not qBH2 It was noted that the BH4 peak obtained following qBH2 injection (Fig 2b) was less than that of the original BH4 standard (Fig 2a) The degree of the reduction was found to be dependent on the time delay between qBH2 generation and injection onto the HPLC column (not shown). The reduction in peak height is likely to be due to the rapid conversion of qBH2 to other products prior to injection onto the HPLC system (Fig. 3) The inclusion of DTE in the mobile phase of the HPLC system therefore means that it is only suitable for the measurement of total BH4+qBH2, as any qBH2 in the sample is immediately converted to BH4

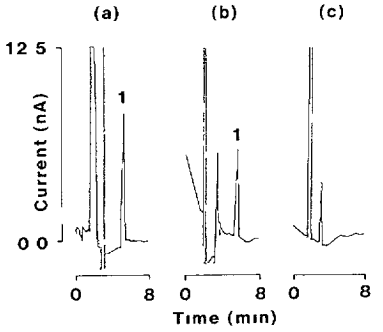

Fig 2 Chromatograms using ED in the redox mode (a) BH4 (32 nmol/l in 10 μmol/l hydrochloric acid) this standard was obtained by two serial dilutions (1 100) in water of a concentrated BH4 solution (320 μmol/l in 0 1 mol/l hydrochloric acid) (b) qBH2 this was generated by the first dilution of the concentrated BH4 (320 μmol/l) into a potassium ferricyanide solution resulting in a ferricyanide/BH4 molar ratio of 2 1, followed by a final dilution into water as above (c) qBH2 was generated as above but with the final dilution in the presence of sodium bisulphite (final concentration, 3 mmol/l) Peak 1 = BH4 Injection volume = 100 μl

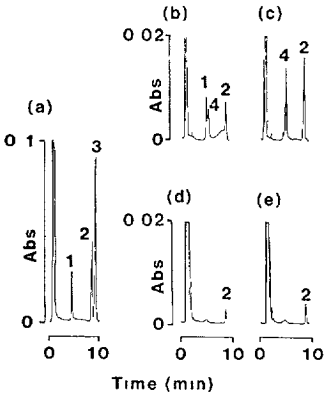

Fig 3 Chromatograms obtained using UV detection at 220 nm (a) BH4 (3 μmol/l), BH2 (4 μmol/l) and B (4 μmol/l), these standards were 1 100 dilutions in 0 05 *M* potassium phosphate buffer, pH 6 8, of concentrated standards (b) Concentrated BH4 standard (320 (μmol/l) was diluted (1 18) in 0 05*M* potassium phosphate buffer containing potassium ferricyanide (ferricyanide/BH4 molar ratio of 2 1) Sufficient buffer was then added to give an overall dilution of the original BH4 standard of 20 This mixture was then immediately injected onto the column (c) Mixture (a) injected 10 min later (d) As for (a) but with the final dilution in the presence of sodium bisulphite (final concentration, 3 mmol/l) (e) Mixture (d) injected 10 min later Peaks 1=BH4+qBH2, 2=BH2, 3=B, 4=PH2

Recently, it has been demonstrated that addition of sodium bisulphite, over a wide pH range, to qBH2 solutions leads to the formation of a stable adduct (qBH2–SO_3) (Fig. 1) [3,7]. Injection of this adduct onto our HPLC system did not result in the detection of a peak at the retention time of BH4 (Fig 2c) demonstrating that the DTE in the mobile phase could not reduce qBH2–SO_3 to BH4 Furthermore, addition of DTE (6 mmol/l) to a solution of qBH2–SO_3 and subsequent HPLC did not result in a measurable BH4 peak (not shown) These results were obtained whether the original qBH2 was formed by ferricyanide or peroxidase–hydrogen peroxide oxidation of BH4

The absence of a peak at the retention time of BH4 suggested that either the adduct was not ED-active at the potentials employed and/or it eluted at a different time point in the chromatogram

We further studied the characteristics of qBH2–SO_3 with the aim of using the adduct formation as a method for determining the qBH2 content of samples The rearrangement of qBH2 in the presence and absence of sodium bisulphite was studied using HPLC with UV detection The system provided good separation of BH4, BH2 and B (Fig. 3a), but did not separate BH4 from qBH2 (Fig. 3b) In the absence of sodium bisulphite there was a rapid decrease in qBH2 and an increase in BH2 and PH2 with time (Fig. 3b and c). This is consistent with the findings of Davis et al. [12] In the presence of sodium bisulphite the qBH2 peak was not present (Fig 3d), indicating qBH2–SO_3 formation The production of BH2 and PH2 with time was prevented by the

addition of sodium bisulphite (Fig. 3d and e) This may reflect the stability of qBH2–SO_3 and/or may be due to the possible adduct formation that can occur between 7,8-dihydropterins and sodium bisulphite [10]

The stability of the adduct was investigated using UV spectroscopy In the absence of sodium bisulphite the unstable nature of qBH2 was clearly demonstrated by the rapid change of its spectrum with time (Fig. 4a) The addition of sodium bisulphite to the qBH2 solution resulted in a stable UV spectrum (Fig 4b).

Direct detection of qBH2–SO_3 by HPLC and UV detection should be possible as this adduct exhibits strong UV absorption at 265 nm (Fig. 4b). However, in our system qBH2–SO_3 was not detected. Adduct formation occurs across the C4a–N5 double bond of qBH2 [3,7] (Fig 1), and at the pH of the mobile phase used (pH 5 2) the molecule would have a negative charge. This charge would lead to a decreased affinity for the column and early elution near or in the solvent front By the use of a different mobile phase, employing an appropriate ion pair, it should be possible to measure directly qBH2–SO_3 by HPLC and UV detection For such a system to be quantitative for qBH2, it would have to discriminate between this adduct and the adducts of BH2 and PH2 which may also be formed [10] following addition of sodium bisulphite to biological samples

To ensure that sodium bisulphite did not react with BH4, sodium bisulphite (3 mmol/l) was added to BH4 solutions Following HPLC and ED, 96% of a 3 2 μmol/l solution was recovered as BH4. The recovery with a 32 nmol/l BH4

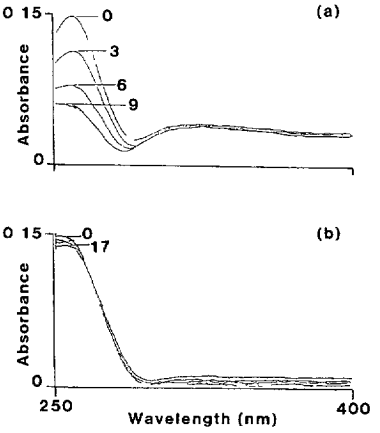

Fig 4 Changes in the UV spectra with time for qBH2 (a) and qBH2-SO_3 (b) qBH2 was generated from BH4 (3 2 μM) by the action of peroxidase (8 μg/ml) and hydrogen peroxide (170 μM) The reaction was carried out in 0 1 mol/l Tris-HCl, pH 7 4 Following qBH2 generation, the change in UV spectrum was monitored at 37°C (a) QBH2-SO_3 was formed by the addition of sodium bisulphite (3 mmol/l) to qBH2 solutions generated as above and the change in the UV spectrum monitored (b) Numbers on spectra indicate time in minutes

solution was 82%. The diminished recovery of BH4 at the lower concentration is attributed to the increased rate of autoxidation, and hence qBH2 formation, which occurs at low BH4 concentrations in the absence of added antioxidants [13].

These results demonstrate that sodium bisulphite reacts with qBH2 and does not react with BH4. The adduct formed with qBH2 is stable, it is not reduced back to BH4 by DTE and it does not chromatograph with BH4. On the basis of the above properties an indirect method for qBH2 measurement was developed based on the principles shown in Fig. 5. The sample is divided into two, and to one half DTE (6 mmol/l) is immediately added. HPLC–ED produces a BH4 peak (*a*), which is derived from the BH4 and qBH2 originally present. To the remaining half, sodium bisulphite (3 mmol/l) is added which reacts with any qBH2 present. DTE (6 mmol/l) is then added immediately after the sodium bisulphite to prevent oxidation of BH4. HPLC procedures a BH4 peak (*b*) which is derived only from the BH4 present originally. It follows that by subtraction of *b* from *a* the qBH2 content may be calculated.

To demonstrate that such a protocol can allow quantitative measurement of qBH2, varying amounts of ferricyanide were added to a solution of BH4 and the qBH2 content determined. The complete oxidation of BH4 to qBH2 by ferricyanide requires a ferricyanide/BH4 molar ratio of 2:1 [6]. Therefore, by altering this ratio, solutions containing known amounts of qBH2 may be rap-

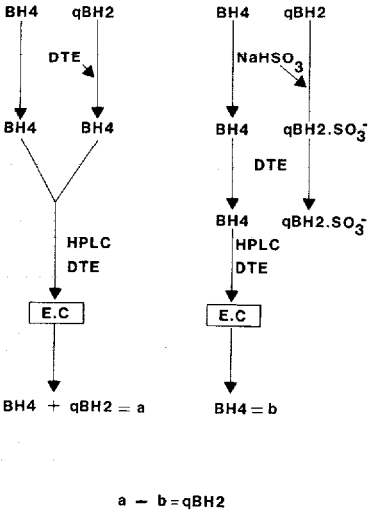

Fig. 5. Measurement of qBH2. The sample is divided. DTE (6 mmol/l, final concentration) is added to one half which is then mixed/homogenised (as necessary). Following deproteinization (if necessary), HPLC–ED measures BH4 and qBH2 as a single BH4 peak (*a*). To the remaining fraction, sodium bisulphite (3 mmol/l, final concentration) is added and, after mixing/homogenisation, DTE is again added. Following deproteinization (if necessary), the BH4 measured is derived only from BH4 present originally (*b*). $qBH2 = a - b$.

idly generated The calculated amount of qBH2 present, as determined by our method, agreed well with the predicted values (Table I)

To ensure that 3 mmol/l sodium bisulphite would be sufficient for total adduct formation in biological homogenates, a large excess of ferricyanide (0.3 mmol/l) was added to a liver homogenate (prepared and treated as described in the legend to Table II). This sample was divided into two, protein was removed from one half by filtration and a sample analysed for qBH2+BH4 content. Sodium bisulphite followed by DTE was added to the other portion. Fol-

TABLE I

MEASUREMENT OF qBH2 FOLLOWING THE REACTION OF BH4 WITH VARYING CONCENTRATIONS OF FERRICYANIDE

Potassium ferricyanide (1 70–6 14 μmol/l) was added to BH4 (3 07 μmol/l, 1 mmol/l hydrochloric acid) The sample was divided and the qBH2 concentrations determined after the sample had been treated as described in Fig 5

Theoretical concentration (μmol/l)	Mean calculated concentration ($n=2$) (μmol/l)
0 00	0 12
0 85	0 80
1 72	1 70
2 57	2 27
3 07	2 92

TABLE II

MEASUREMENT OF qBH2 IN BIOLOGICAL SAMPLES

Samples (0 5 ml) of lumbar CSF, from children with a range of disorders, excluding defects of pterin metabolism, were collected into plain plastic tubes The tubes were stoppered placed immediately on dry ice and stored at −70 °C until assayed For assay, samples were thawed and 100 μl injected onto the HPLC system and BH4 was measured (*a*) Sodium bisulphite (3 mmol/l final concentration) was added to an additional 100 μl which was refrozen in liquid nitrogen until assayed Following thawing and HPLC, BH4 was measured (*b*) Rat liver was immediately removed and placed in liquid nitrogen and stored at −70 °C A fraction of liver was homogenised (5%, w/v) in 0 1 mol/l citrate buffer (pH 4 0), containing DTE (6 mmol/l) Protein was removed by centrifugation through a Millipore cellulose filter (Exclusion, 10 000 nominal molecular mass) and BH4 measured (*a*) A second fraction was homogenised in the presence of sodium bisulphite (3 mmol/l), followed by DTE addition (6 mmol/l) BH4 was measured, after protein removal (*b*) % qBH2 = $(a-b)/a \times 100$

Sample	Concentration of BH4+qBH2 (mean ± S D)	n	Percentage qBH2
CSF	39 1 ± 16 6 pmol/ml	7	11–40
Rat liver	5 12 ± 1 01 nmol/g wet weight	4	17–20

lowing protein removal and HPLC analysis the qBH2 content was calculated to be 94% of the total BH4 + qBH2 present

At present the amount of qBH2 present within biological samples is unknown As a preliminary study to address this point, the qBH2 content of human cerebrospinal fluid (CSF) and of rat liver was determined The qBH2 concentration of CSF and rat liver ranged from 11 to 40% and 17 to 20%, respectively, of the total BH4 + qBH2 present (Table II). Currently it is uncertain whether the qBH2 detected arose from autooxidation of BH4 following collection and storage or represents the true endogenous qBH2 Further studies are in progress to address these questions

The method described here provides a simple and specific method for the indirect measurement of qBH2 and represents the first quantitative method for the analysis of qBH2 in biological samples

REFERENCES

1 S Kaufman, in E Usdin, N Weiner and M B H Youdim (Editors), Function and Regulation of Monoamine Enzymes, MacMillan New York, 1981, p 165
2 J E Craine, S E Hall and S Kaufman, J Biol Chem , 247 (1972) 6082
3 S Matsuura, S Murata and T Sugimoto, in B A Cooper and V M Whitehead (Editors), Chemistry and Biology of Pteridines, Walter de Gruyter, Berlin, 1968, p 81
4 J Haavik and T J Flatmatk, J Chromatogr , 257 (1983) 361
5 C E Lunte and P T Kissinger, Anal Chim Acta, 158 (1984) 33
6 C E Lunte and P T Kissinger, J Chromatogr , 317 (1984) 407
7 S Matsuura, S Murata and T Sugimoto, Tetrahedron Lett , 27 (1986) 585
8 D W Howells, I Smith and K Hyland, J Chromatogr , 381 (1986) 285
9 J E Ayling and S W Bailey, in H Ch Curtius, W Pfleiderer and H Wachter (Editors), Biochemical and Clinical Aspects of Pteridines, Vol 2, Walter de Gruyter, Berlin, 1983, p 147
10 W L F Armarego, D Randles and H Tagucha, Eur J Biochem , 135 (1983) 393
11 S J R Heales and K Hyland, Pteridines, submitted for publication
12 M D Davis, S Kaufman and S Milstien, Eur J Biochem , 173 (1988) 173
13 D W Howells and K Hyland, Clin Chim Acta, 167 (1987) 23