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Note

Post-column oxidation of reduced biopterins for fluorescence detection

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It has been observed that part of phenylketonuria, Parkinsonism and tryptophan metabolism disorder is not caused by a low activity of metabolic enzymes, but by a deficiency of their cofactor, 5,6,7,8-tetrahydrobiopterin (BH_4). In fact, oral or intravenous administration of BH_4 decreased the phenylalanine concentration in the blood of patients with phenylketonuria who were considered to be BH_4 deficient[1,2]. Therefore, measurement of blood levels of BH_4 and its precursors, *i.e.*, biopterin (B), 7,8-dihydrobiopterin (BH_2) and sepiapterin (Sp), is important for typing of the diseases. Furthermore, fractional analysis of naturally formed BH_4 (*R*-BH_4, with an *R* configuration at position 6 of the pteridine ring) and unnaturalformed BH_4 (*S*-BH_4) is now needed in order to establish suitable clinical dosages of chemically synthesized BH_4 (a mixture of *R*- and *S*-BH_4), because a difference between *R*- and *S*-BH_4 with respect to activity and metabolism is expected.

A method for the separation of B, BH_2, *R*-BH_4 and *S*-BH_4 was reported by Bailey and Ayling[3], who used a Partisil-10 SCX column. Also, the fractional analysis of B of Sp using the same column has been reported[4]. However, it appears that the separation could be improved. Three methods for post-column detection have been reported, *viz.*, a UV detection method for all of the substances[3], a fluorescence detection method for B and Sp[4] and an electrochemical detection (ElCD) method[5] for BH_2, *R*-BH_4 and *S*-BH_4. The UV method has both low sensitivity and low selectivity. The fluorescence and ElCD methods do not permit the analysis of either oxidized pterins (B and Sp) or reduced pterins (BH_2, *R*-BH_4 and *S*-BH_4).
We have therefore developed a post-column oxidation method for fluorescence detection, in which reduced biopterins are oxidized to B after the column separation and the fluorescence is detected. Using this method, B, Sp, BH_2, *R*-BH_4 and *S*-BH_4 can be analysed simultaneously with high sensitivity.

MATERIALS AND METHODS

Crystal of B was kindly provided by Dr. Noguchi (The Biomedical Institute, Suntory KK, Osaka, Japan). BH_2 and a mixture of *R*- and *S*-BH_4 were prepared by

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reduction using zinc dust in potassium hydroxide solution and by catalytic hydrogenation with platinum(IV) oxide as a catalyst, respectively, according to the methods of Kaufman[6]. They were used without purification, although they contained a small amount of B. Sp was prepared by oxidation of BH_4 mixture with iodine in alkaline iodide solution, followed by fractionation by liquid chromatography (LC) as described below. The other chemicals used were of analytical-reagent grade.

Biopterins were separated using a 25 cm × 4.6 mm I.D. Partisil-10 SCX column (Whatman, Clifton, NJ, U.S.A.) at 20°C with an eluent consisting of 15 m*M* ammonia–formic acid (pH 3.20) containing 0.1 m*M* EDTA at a flow-rate of 1.0 ml/min. Phosphoric acid could be used in place of formic acid in the eluent, but acetic acid was unsuitable because of lower resolution, perhaps, caused by the higher viscosity of the eluent. EDTA was incorporated in the eluent because iron ions especially oxidize BH_4 to BH_2 in the presence of trace amounts of dissolved oxygen. Nitrogen had been bubbled through the eluent to remove dissolved oxygen prior to use. The fluorescence of B and Sp was detected with an FLD-1 fluorimeter (Shimadzu Seisakujo, Kyoto, Japan) equipped with a 360-nm lamp and an EM-4 cut-off filter.

RESULTS

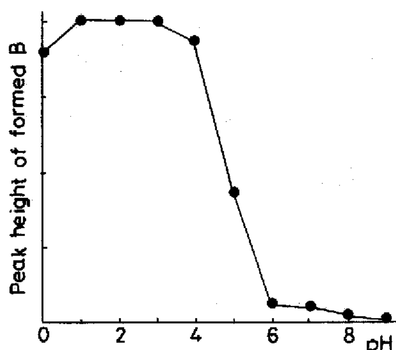
The conditions for the oxidation of BH_4 to B were investigated, using a manual method followed by fluorescence detection of B with LC. BH_4 was incubated with oxidants in acetate buffer (pH 4) or borate buffer (pH 9). As shown in Table I, sodium nitrite was the best agent in the acidic buffer, because the reaction rate was high and decomposition of B was negligible. Sodium hypochlorite and potassium periodate were unsuitable, because the oxidation was accompanied by decomposition into less fluorescent substances. On the other hand, in alkaline buffer potassium periodate gave the highest recovery of Sp although a small decomposition peak was observed. Sodium nitrite had a very low oxidizing activity, and sodium hypochlorite decomposed biopterins. Thus, oxidation with sodium nitrite in an acidic buffer was chosen.

TABLE I

B OR Sp FORMATION FROM BH_4 MIXTURE BY OXIDANT

16 µl of BH_4 mixture were incubated with oxidant at 50°C for 5 min, and analysed by LC using an FLD-1 detector.

Oxidant	*Concentration (mM)*	*Amount formed (peak height)*	
		B (pH 4)	*Sp (pH 9)*
$NaNO_2$	(2.0)	17.8	2.8
$NaNO_3$	(2.0)	4.2	1.8
H_2O_2	(10.0)	3.7	0.4
KIO_4	(1.0)	1.8	15.0
NaOCl	(0.2)	11.9	0.7
I_2	(0.2)	7.2	9.0
$NaClO_3$	(4.0)	1.8	0.1
$NaBrO_3$	(4.0)	5.3	0.9
$NaIO_3$	(4.0)	4.8	0.2
None		0.3	1.9



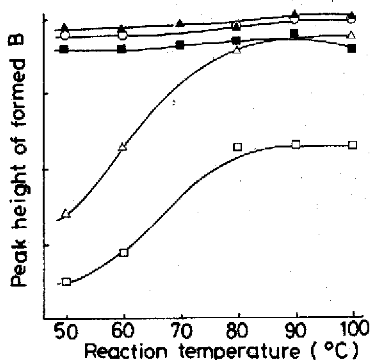
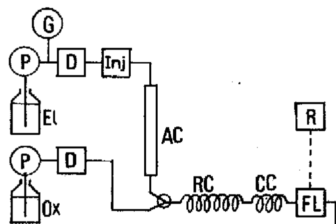


Fig. 1. Effect of pH on oxidation of BH_4. BH_4 mixture (16 μM) was incubated with 2 mM sodium nitrite at 50°C for 5 min in a buffer of hydrochloric acid (pH 1), formate (pH 2 and 3), acetate (pH 4, 5 and 6) or phosphate (pH 7, 8 and 9).

Fig. 2. Effect of nitrite concentration and reaction temperature on oxidation of BH_4. BH_4 mixture (2 μM) was incubated in formate buffer (pH 3.0) with 0.003 mM (□), 0.03 mM (△), 0.3 mM (○), 1.0 mM (▲) and 10 mM (■) sodium nitrite for 3 min at the indicated temperatures.

The amount of B formed by nitrite oxidation of BH_4 reached a plateau after about 2 min under the conditions given in Table I. The reaction was fast below pH 5, as shown in Fig. 1, which suggests that the oxidizing substance is nitrous acid because its pK_a is 3.4.

The effects of nitrite concentration and reaction temperature were investigated next. For reaction at pH 3 for 3 min, a temperature of more than 80°C was required with 0.03 mM sodium nitrite; the reaction went well at 50°C or more with 0.1–10 mM nitrite, as shown in Fig. 2. However, reaction at 100°C with 10 mM nitrite gave a low conversion.



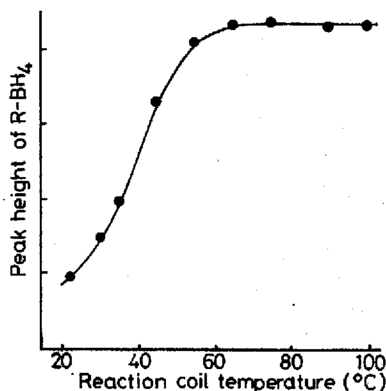


Fig. 3. LC system with nitrite oxidation. P = pump; G = pressure gauge; D = damper; Inj = sample injector; AC = analytical column; El = eluent (1.0 ml/min); Ox = oxidant solution (5 mM sodium nitrite, 0.25 ml/min); RC = reaction coil (5 m × 0.5 mm, PTFE tube); CC = cooling coil (3 m × 0.25 mm, PTFE tube, at 20°C); FL = FLD-1 detector; R = recorder.

Fig. 4. Effect of reaction coil temperature on peak height of BH_4.

Based on these data, it was decided to perform the post-column reaction at pH 4 or below with a final concentration of 1.0 m*M* sodium nitrite at 60–90°C for 1–2 min. The system is illustrated in Fig. 3. The eluate from the column was mixed with one quater of its volume of 5 m*M* sodium nitrite solution and the mixture was heated in a reaction coil for 1 min, cooled and led to the fluorescence detector. The nitrite solution was not buffered, because the eluate was buffered to pH 3.2 in the separation system. A temperature of 65–100°C was found to be suitable for the reaction (Fig. 4), so 80°C was employed as the mean. The calibration graph for *R*-BH_4 (Fig. 5) was linear in the range 10–400 pmol of BH_4 mixture injected, which was composed of *R*-BH_4 (*ca.* 60 %), *S*-BH_4 (*ca.* 30 %) and others. The detection limit was 0.17 pmol for B and 0.7 pmol for *R*-BH_4 (injected amounts) calculated as three times the signal to noise ratio.

BH_2, in addition to BH_4, was quantitatively oxidized to B in this system. Alkali-treated BH_4 gave a broad and very low peak distinguished from *R*- and *S*-BH_4, which indicates that dihydrosepiapterin was difficult to oxidize with nitrite.

Further the height of each peak increased about 3-fold when the reaction mixture was brought to pH 9.5–10.0 by mixing it with alkaline borate solution before the cooling coil, as shown in Fig. 6. The detection limit was 0.06 pmol for B and 0.25

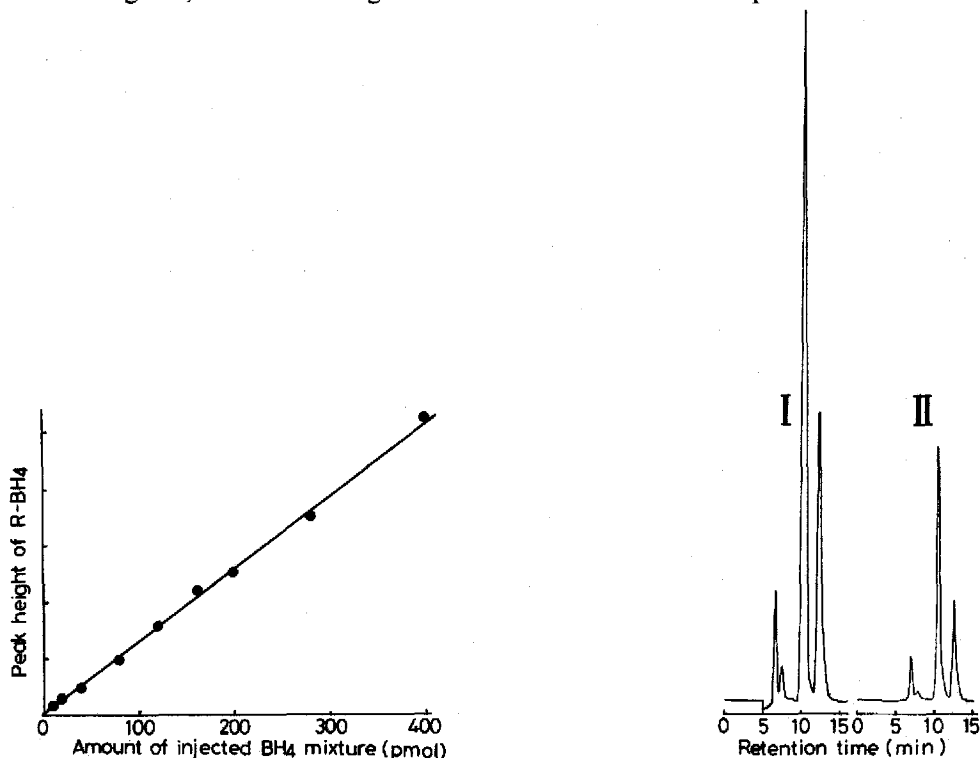


Fig. 5. Calibration graph for *R*-BH_4. The reaction coil temperature was set at 80°C.

Fig. 6. Effect of alkalinization after oxidation on peak heights. BH_4 mixture (80 pmol) was injected. The major peaks were B, BH_2, *R*-BH_4 and S-BH_4, from left to right. I, Alkalinization by mixing of 0.4 *M* boric acid plus potassium hydroxide at a rate of 0.3 ml/min before the cooling coil to give a final pH of 9.7; II, control.

pmol for R-BH_4 (injected amounts). This effect occurs because the fluorescence intensity of B is about four times higher at pH 10 than at pH 3–7.

DISCUSSION

We selected sodium nitrite as an effective oxidant in acidic solution to convert BH_2 and BH_4 into B. Iodine, which is commonly used to oxidize BH_2 and BH_4[7], was found to be a weaker oxidant than nitrite and to quench the fluorescence. Although periodate was too strong for oxidation of biopterins, further examination of this oxidant in alkaline solution is desirable. For the present, the oxidation method with nitrite is considered to be suitable for analysing biopterins, because their separation is generally effected in acidic media, and the range of reaction conditions (nitrite concentration, pH, temperature and reaction time) that gave the same result is wide.

By using nitrite oxidation followed by alkalinization, the detection limit was 15 pg for B and 60 pg for *R*-BH_4 (injected amounts). These values are lower than those obtained by UV detection (10 ng for R-BH_4)[8] or the ElCD method (200 pg for BH_2 and *R*-BH_4)[5]. Hence our method seems to be more sensitive superior than the other detection methods for the analysis of reduced biopterins.

For protecting biopterins from oxidation during a column separation, use of an eluent free from oxygen, prepared by bubbling nitrogen gas through it, is required. As an alternative, we examined the use of an eluent containing an antioxidant, and found that ascorbic acid is suitable as it prevents the oxidation of biopterins, leaving the post-column nitrite oxidation reaction unaffected. The details will be reported elsewhere.

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