3′-HYDROXYSEPIAPTERIN IN PATIENTS WITH DIHYDROBIOPTERIN DEFICIENCY

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1. Introduction

In recent years it has been shown that the group of diseases known as phenylketonuria includes very rare and severe diseases caused by defects in biopterin metabolism [1–6]. These findings have focused interest on a new area of research that may also have great significance for neuroscience and future treatment of affective disorders. Tetrahydrobiopterin (BH_4) is a cofactor of the monooxygenases phenylalanine-4-hydroxylase, tyrosine-3-hydroxylase and probably also tryptophan-5-hydroxylase, key enzymes in dopamine and serotonin production. Biopterin is formed from guanosine triphosphate in several steps, some of which are not known exactly. We report here the detection of 3′-hydroxysepiapterin (HS) in urine from a patient with defective dihydrobiopterin (BH_2) biosynthesis. This finding could be of value for the understanding of biopterin biosynthesis and the more exact localization of the enzyme defect in biopterin-deficient patients.

2. Materials and methods

2.1. *Patients with dihydrobiopterin deficiency*

Z. Y., female, born in September 1976: Case report in [7]. B. E., female, born 3 May 1979. A detailed case report will be published in [8]. In both patients, the metabolic defect lies between dihydroneopterin triphosphate and L-sepiapterin [5,6]. Z. Y. and B. E. need BH_4 dihydrochloride in daily doses of 1.25 and 2.5 mg/kg, respectively, to keep their plasma phenylalanine levels normal when on a diet unrestricted in protein.

2.2. *Materials*

Urine portions were stored frozen without any additives. Samples were protected from direct light. Pterin-6-carboxylic acid (P6C) was from Fluka AG (Buchs SG); other pterins except for HS, were obtained from Professor M. Viscontini, University of Zurich, and Dr B. Schircks, CH-8623 Wetzikon.

2.3. *3′-Hydroxysepiapterin (6-(1′-oxo-2′,3′-dihydroxypropyl)-7,8-dihydropterin (HS)*

This was prepared analogously to L-sepiapterin (S) [9] as follows. Tetrahydroneopterin dihydrochloride, 12 mg (36 μmol) in 2 ml 0.15 mol/l sodium acetate (pH 4.1) was kept for 5 days in an open 25 ml round-bottom flask in the dark. After evaporation to dryness, the residue was redissolved in water at 60°C and chromatographed on a 1.6 × 28 cm column of cellulose in water at a 50 ml/h flowrate. The main yellow zone eluted between 105–150 ml and was rechromatographed on a 1.6 × 25 cm column of Sephadex G-15 in water. The eluate between 76–106 ml was evaporated to dryness, the residue dissolved in warm absolute ethanol and precipitated with diethylether to give chromatographically pure HS. The yield was ~2 mg.

2.4 *Isolation of the unknown compound from urine of patient B. E.*

Urine, 50 ml, creatinine conc. 18 mg/dl, was concentrated to ~10 ml in vacuo, centrifuged and the clear supernatant chromatographed on a 1.6 × 28 cm column of Sephadex G-15 in water. The eluate between 80–110 ml was collected. Two further 50 ml urine portions were treated similarly and the corresponding yellow fractions combined for rechromatography on a 1.6 × 25 cm column of cellulose in water.

The eluate between 125–158 ml was collected, concentrated in vacuo and stored frozen.

2.5. *Derivatives*

Methoxime derivatives were prepared in absolute pyridine at 60°C for 3 h, using methoxyamine hydrochloride, 2% in pyridine. Trimethylsilyl derivatives were prepared in bistrimethylsilyl-trifluoroacetamide (1:1, v/v) for 1 h at 100°C. Reduction/oxidation of aqueous HS or S, respectively, was performed with $NaBH_4$ for 1 min followed by adjustment to pH 2 with HCl, shaking with MnO_2 for 5 min and centrifugation.

2.5. *High-performance liquid chromatography (HPLC)*

Samples were filtered through SEP-PAK C_{18} cartridges (Waters Assoc., Milford, MA) using 20 ml water. HPLC was performed on a 4.5 X 250 mm column of 7 μm Lichrosorb RP-8 (Knauer, Berlin) using acetic acid–water (1:2000, v/v) as solvent at 44°C and fluorimetric detection at 350/450 nm (oxidized, blue fluorescing pterins) or 420/530 nm (HS, S). Gas chromatography–mass spectrometry (GC–MS) was performed on a 20 m SE-52 glass capillary column (H. Jaeggi, Trogen) at 175–250°C, 6°C/min, He 1 bar with direct coupling to a mass spectrometer (F-16, Vacuum Generators, Altrincham) and an INCOS data system (Finnigan Instr., Sunnyvale, CA). All spectra were taken at 20 eV. High-voltage electrophoresis (HEP) was performed at 100 V/cm in acetic acid–formic acid–water (120:26:850, v/v/v) pH 1.9 on Schleicher and Schüll paper no. 2043 B.

3. Results

In urine of patients Z. Y. and B. E., a yellow fluorescing spot was visible on paper high-voltage electrophoresis (HEP) with a low mobility very similar to S at pH 1.9, but with a lower migration rate than S in paper chromatography in water or in 1-butanol–acetic acid–water solvents. The isolated yellow compound showed a UV spectrum ($\lambda_{max}^{H_2O}$ 216, 268 and 424 nm with log ε 4.21, 4.22 and 4.02, respectively) and fluorescence spectrum ($\lambda_{max}^{H_2O}$ 420/530 nm) similar to S. It gave threo- and erythro-neopterin (Ne) in a ratio of 2.0:1 after reduction/oxidation as did synthetic HS; whereas S gave a threo-/erythro-biopterin ratio of 1.34:1 under the same conditions, measured by HPLC and checked by MS. The isolated compound could be degraded to pterin-6-carboxylic (P6C) acid by treatment with $KMnO_4$ or MnO_2. The isolated compound could not be distinguished chromatographically from synthetic HS, neither as the free compounds nor as the methoxime-7,8-dehydro derivatives in HEP at pH 1.9 and 100 V/cm, the relative mobilities (uncorrected for electroendosmosis) of HS, 7,8-dehydro-HS-methoxime, S, 7,8-dehydro-*S*-methoxime, B, Ne, P6C and Xanthopterin (X) were 12, 52, 16, 62, 100, 96, 21 and 50, respectively. In paper chromatography in 1-butanol–acetic acid–water (20:3:7, v/v/v), the R_F X 100 values of these compounds were 19, 37, 33, 55, 20, 33, 20 and 31, respectively. HS, Ne, B, X eluted in HPLC on RP-8 at 44°C after 10.8, 4.89, 8.94 and 6.30 min, respectively. The number of trimethylsilyl (TMS) groups of the derivatives of HS, 7,8-dehydro-HS-methoxime, B and X were 5, 4, 4 and 3, and these compounds eluted in GC on a SE-52 glass capillary after 590, 607, 427 and 249 s, respectively.

The isolated compound gave an electron impact (20 eV) mass spectrum identical to that of authentic HS $(TMS)_5$enol with signals at *m/e* (% rel. intensity) 613 (M^+, 100), 598 (12), 541 (4), 523 (9), 510 (6), 495 (3), 449 (3), 409 (3), 408 (3), 381 (8), 380 (7), 307 (2), 205 (2), 147 (5). Isolated and synthetic compounds gave a small additional GC peak of HS $(TMS)_6$ eluting at 620 s. More structural information was given by the electron impact (20 eV) mass spectra of the methoxime derivatives.

7,8-Dehydro-HS-methoxime $(TMS)_4$ showed main signals at *m/e* 568 (M^+, 23%), 553 (17), 537 (28), 478 (69), 465 (6), 447 (42), 332 (37), 317 (52), 204 (100), 189 (30), 147 (33), 133 (29), 117 (50). The ion at *m/e* 204 (base signal) is interpreted as TMS–O–CH = CH–OTMS resulting from side chain cleavage, which also leads to CH_3OH and 6-cyanopterin $(TMS)_2$, the latter giving strong signals at *m/e* 332 and 317. An analogous fragmentation pattern was observed in MS of 7,8-dehydrosepiapterin methoxime $(TMS)_3$.

Using synthetic HS as standard, several urine specimens were analyzed by HPLC at 420/530 nm after prepurification on Sep-Pak C_{18}. When concentration was expressed in mmol/mol creatinine, values of 3.9, 9.65, 5.64, 5,54 and 7.54 were found in urine specimens of patient B. E., B. E., B. E., Z. Y., and Z. Y., respectively, and 0.17, 0.07 and 0.02 in 3 urines from

healthy controls. Only traces of HS were found in urine of patients with dihydropteridine reductase deficiency.

4. Discussion

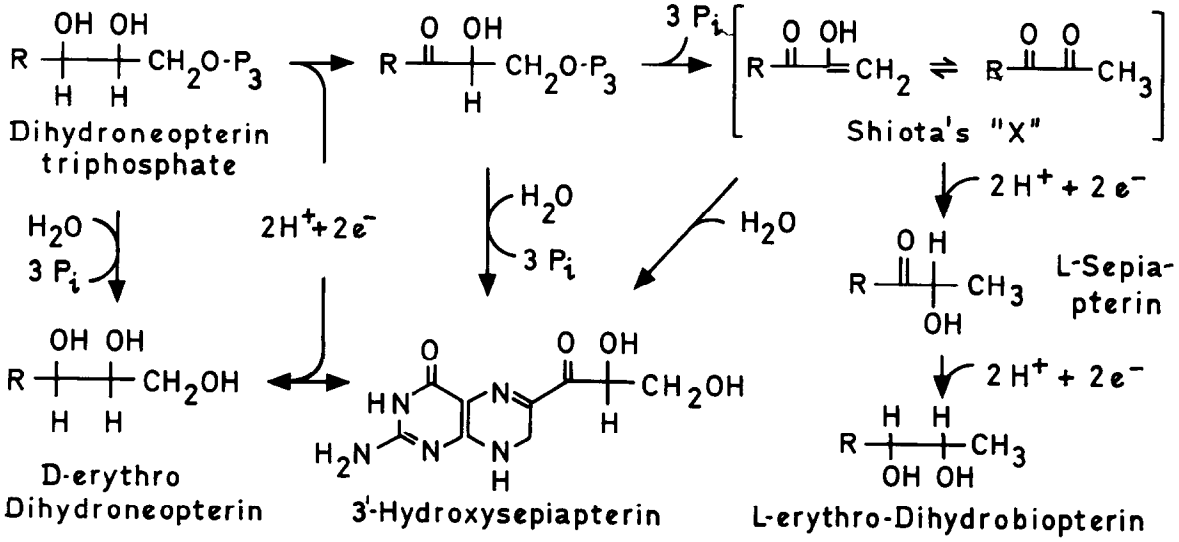
From these results we conclude that the isolated compound is HS. It is present in fresh, native urine, as was shown by HEP. HS has been described in [10] as one of the products formed during reoxidation of dihydroneopterin by air. We found that HS is formed in high yield from NeH_4 at pH 4 under the same conditions as S is formed from BH_4 [9].

The origin of urinary HS is not yet known; three possible ways of formation are shown in scheme 1. It may arise at least partially from dihydroneopterin by enzymic or nonenzymic oxidation, even during the standing of urine before analysis. Then, one would also expect elevated urinary S when BH_2 is elevated, as in patients with dihydropteridine reductase deficiency. However, we could never observe this. At room temperature, HS was formed only slowly from NeH_4 added to normal human urine. Furthermore, a urine portion from patient B. E., collected directly on dry ice and kept deep frozen until just before analysis, contained 3.9 mmol HS/mol creatinine.

This may indicate that HS is of endogenous origin and is a normal metabolite in man. One might even speculate that urinary dihydroneopterin could originate from HS by enzymic reduction. As shown in scheme 1, HS could arise from dihydroneopterin triphosphate (NeH_2P_3), a well known intermediate in biopterin biosynthesis [11–13]. NeH_2P_3 was claimed to be converted in rat brain into dihydrobiopterin (BH_2) by a single enzyme, called BH_2-synthetase [14]. However, in kidneys of Syrian golden hamster [15] and chicken [18], at least 2 enzymes were necessary for this reaction. Under certain conditions, S was a free intermediate [13,15,16]. In [13] A chicken kidney enzyme preparation 'A_2' was obtained which transformed NeH_2P_3 into an unknown intermediate (Shiota's X) with a postulated structure of 6-(1′-2′-dioxopropyl)-7,8-dihydropterin. The triphosphate of HS would fit into this postulated pathway (scheme 1) as the precursor of the dioxo-compound (by elemination of inorganic P_3 for BH_2 biosynthesis) and of HS (by hydrolysis of the triphosphate ester, 'overflow pathway'). The observed accumulation of urinary HS in patients with BH_2-deficiency could mean that they are unable to eliminate the triphosphate group in the normal way. Instead, the overflow pathway of hydrolysis by a (tri)phosphatase may occur. A third possible but less likely pathway leading to HS would be hydration of 'Shiota's X' (scheme 1).

Scheme 1

Postulated steps in dihydrobiopterin biosynthesis and possible pathways for formation of 3′-hydroxysepiapterin



R = 7,8-dihydropterin-6-yl

Increased urinary HS in addition to elevated Ne may be used for diagnosis of a defect in BH_2 biosynthesis. Analysis by HPLC with fluorimetric detection at 420/530 nm is very simple and highly specific.

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