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Short Communication

7-Substituted Pterins: Formation in Carbinolamine Dehydratase Deficiency

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Introduction

A new transient form of hyperphenylalaninemia is characterized by the excretion of 7-substituted pterins in urine of patients, e. g. the 7-L-analogue of L-biopterin (L-primapterin), the 7-D- or L-analogue of D-neopterin (D- or L-anapterin), and 6-oxo-7-L- or D-analogue of 7-oxo-L-biopterin (6-oxo-L- or D-primapterin) (1, 2).

The structure elucidation of the 7-substituted pterins was performed using GC-MS, oxidation to their 7-carboxylic acids, and comparison with reference substances (1, 3). The determination of the absolute configuration of excreted primapterin belonging to the L-series like its 6-analogue was done by CD-measurement (4). Both, L-primapterin and D- or L-anapterin were identified in control urines, blood and in liver of man and mouse (3, 5), but only in very small amounts.

The excretion of 7-substituted pterins seems to be associated with an enzyme defect in the aromatic amino acid hydroxylating system. *In vivo* formation of 7-substituted pterins was achieved incubating their fully reduced 6-analogues with pterin-4a-carbinolamine dehydratase-free rat or human phenylalanine hydroxylase (6, 7). It was concluded that the patients most probably suffer from a pterin-4a-carbinolamine dehydratase (PCDH) deficiency (6).

From the finding that *in vitro* the 7-D- or L-analogue of D-neopterin originates from its 6-tetrahydro analogue by incubating D-NH_4 with dehydratase-free phenylalanine hydroxylase, one might speculate that *in vivo* D-tetrahydroncopterin also acts as cofactor for the pterin dependent aromatic amino acid hydroxylases. This might be the reason for the occurrence of D- or L-anapterin in the urine of patients and controls.

In this study we show that D-NH_2 is a substrate for DHFR and can be transformed to D-NH_4 which possibly could also be a cofactor for the aromatic amino acid hydroxylases *in vivo*. Further we provide direct GC-MS evidence for the structure of *in vitro* formed L-primapterin. We could give an explanation for the hyperphenylalaninemia of these primapterinuric patients.

Methods

Pterins were oxidized with manganese dioxide under acidic conditions [8]. They were analyzed by reverse-phase HPLC with fluorescence detection (excitation/emission wavelengths were 350/450 nm). Inhibition experiments with dehydratase-free rat liver phenylalanine hydroxylase were performed in 0.1 M Tris-HCl, with 0.35 U phenylalanine hydroxylase (specific activity 8.5 U/mg protein), 1 mM L-phenylalanine, and an excess of both catalase and DHPR, 5 mM NADH, L-tetrahydrobiopterin (L-BH_4) as substrate (concentrations from 5 to 20 µM) and L-tetrahydroprimapterin as potential inhibitor (concentrations from 2 to 30 µM). For GC-MS measurements of the *in vitro* formed L-primapterin we used a *VG-16F* single-focusing magnetic-field instrument according to the method of Kuster et al. (9).

Results and Discussion

Structure elucidation of in vitro *formed L-primapterin*

The identiy of L-primapterin was confirmed by GC-MS as TMS derivative. For GC a glass capillary column was used. Under the GC conditions used the silyl derivatives of the two isomers are clearly separated (see Fig. 1).

The mass spectra of both peaks were essentially identical and were confirmed using chemically synthesized L-biopterin and L-primapterin. The signal at m/z 409 corresponds to the pterin nucleus (Fig. 1) when the 1′-2′ bond of the L-primapterin side-chain is cleaved and a proton of the 3′-methyl group shifts to the 6-position of the pyrazine ring [9]. GC-MS data obtained of *in vitro* formed L-primapterin give conclusive evidence for the proposed structure.

Inhibition of phenylalanine hydroxylase

The reason for transient hyperphenylalaninemia of primapterinuric patients has for a long time been a puzzling problem. L-tetrahydroprimapterin might inhibit phenylalanine hydroxylase activity, resulting in an increase of plasma phenylalanine. We showed that L-tetrahydroprimapterin competes with L-BH_4 for the active site of the enzyme (Fig. 2). The K_i value of 8 μM obtained for L-tetrahydroprimapterin compares to K_m values of 20 μM and 1.5 μM, respectively, for L-tetrahydroprimapterin and L-BH_4 as substrates. This observation strongly suggests that in these patients phenylalanine hydroxylase is inhibited by their own abnormal metabolites, the 7-substituted tetrahydropterins, resulting in hyperphenylalaninemia.

The group of Davis et al. (10) states another reason for the hyperphenylalaninemia of these patients: L-tetrahydroprimapterin might be an inefficient cofactor of the phenylalanine hydroxylase and the oxidation of the pterin cofactor would not be coupled to the amino acid hydroxylation.

Nevertheless, primapterinuric patients excrete less L-BH_4 compared to controls (about 40% of urinary L-biopterin in tetrahydroform compared to 60–80% in controls). Still, there should be enough cofactor present, especially because regeneration of cofactor with DHPR was not found to be inhibited by 7-substituted pterins.

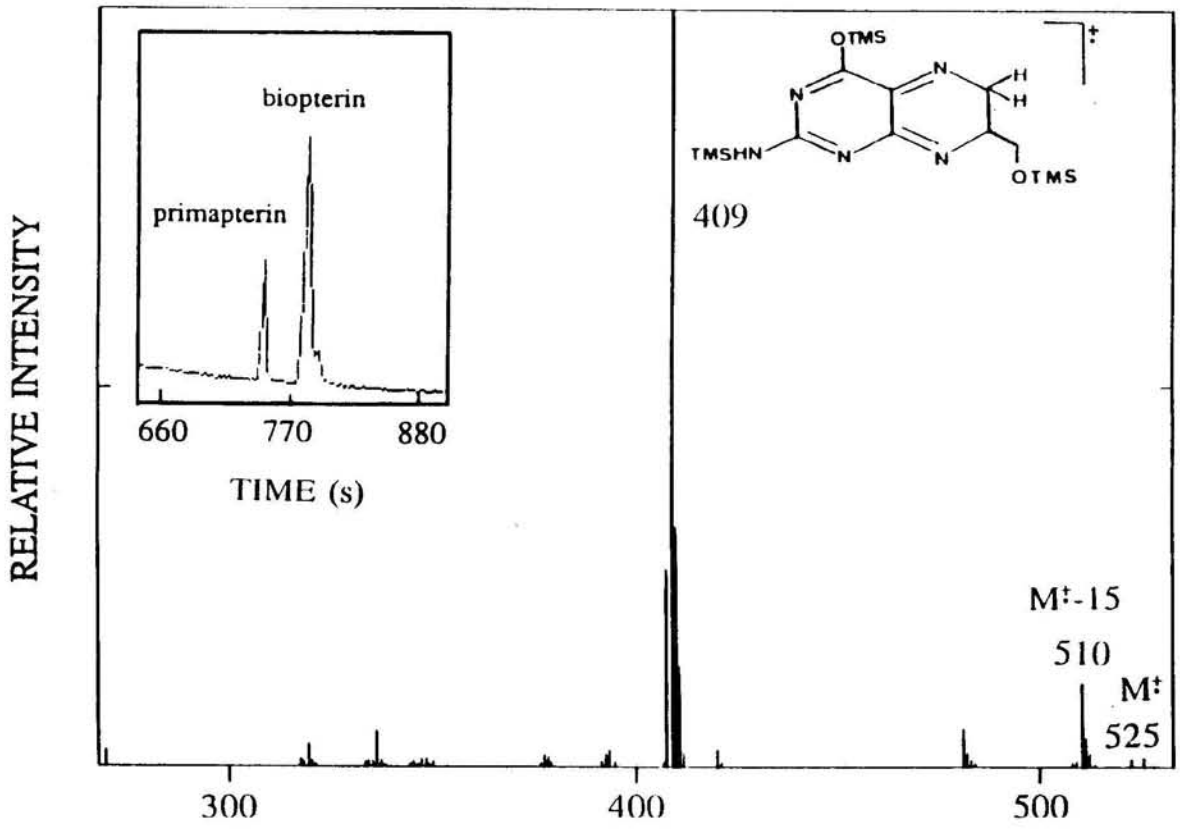

Figure 1. Gas-chromatographic analysis of derivatized L-biopterin and L-primapterin, and MS fragmentation pattern of L-primapterin. The insert represents the GC-MS TIC profile and the figure the electron impact mass spectra of the peak corresponding to L-primapterin.

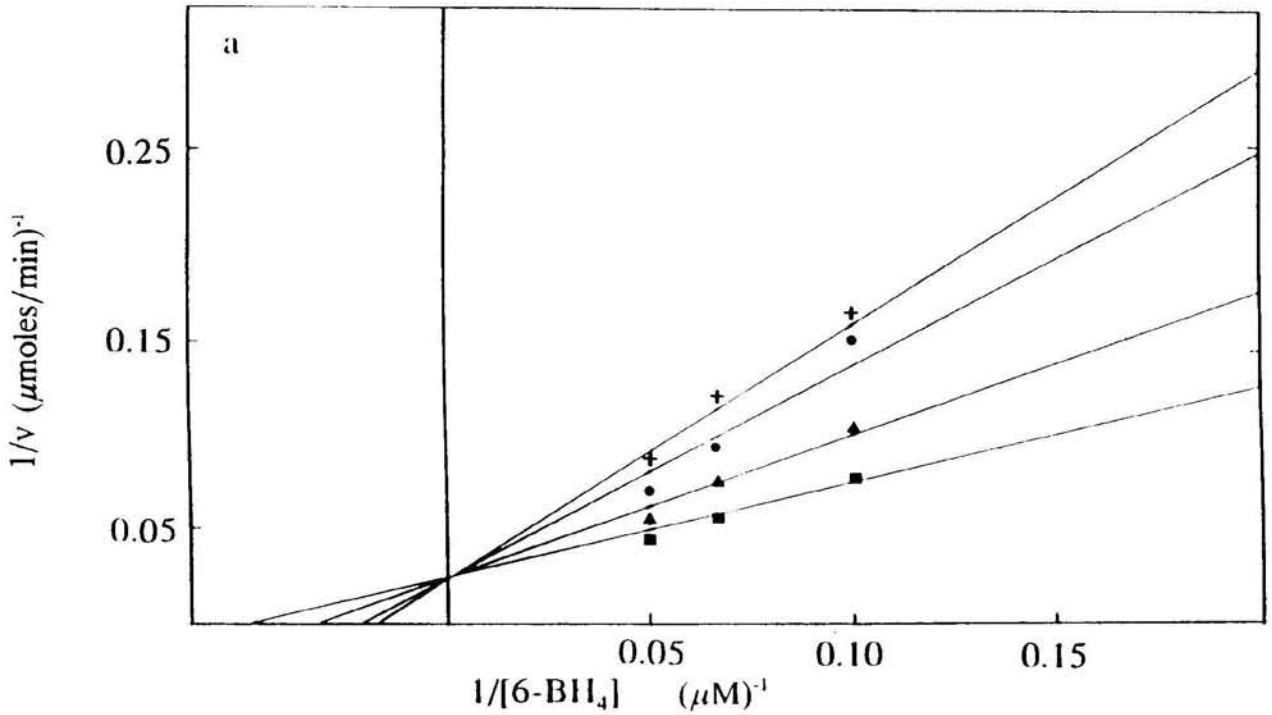

Figure 2. Inhibition of the phenylalanine hydroxylase reaction by L-tetrahydroprimapterin. Determination of inhibition type. Lineweaver Burke plot at varying concentrations of inhibitor: 0 μM up to 20 μM L-tetrahydroprimapterin.

D-Tetrahydroneopterin as cofactor in vivo

As the 7-D- or L-isomer of D-neopterin is also excreted in the urine of primapterinuric patients as well as in control urine one can speculate that other tetrahydropterins than L-BH_4 temporarily act as cofactors and that the need for them varies at different stages of development. A metabolic intermediate in BH_4 biosynthesis is D-tetrahydroneopterin triphosphate a compound that is present in substantial concentrations in body fluids. The phosphate group can be hydrolysed by the action of phosphorylases. DHFR, which accepts a variety of substrates, may also reduce D-NH_2 to its biologically active tetrahydroform. *In vitro* we found substantial concentrations of D-tetrahydroneopterin after incubation of D-dihydroneopterin with dihydrofolate reductase *in vitro*.

Since *in vitro* D-tetrahydroneopterin is a cofactor of phenylalanine hydroxylase, and in spite of the fact that the activity is only 20% compared with L-BH_4, this pterin might play an important role in patients with 6-pyruvoyl tetrahydropterin synthase deficiency. These patients excrete increased amounts of D-neopterin but very low levels of L-biopterin D-tetrahydroneopterin might thus act as cofactor for the aromatic amino acid hydroxylases. Here we might have an explanation for the finding that these patients have low but nevertheless substantial levels of the neurotransmitters deopamine and serotonin in tissues and bodyfluids.

Acknowledgement

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