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LC determination of biopterin reduced forms by UV-photogeneration of biopterin and fluorimetric detection

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ABSTRACT

An off-line photoirradiation LC fluorimetric method to determine tetrahydrobiopterin (BH_4), by photogeneration of biopterin (BIO), is described, as an alternative way to the chemical oxidation procedure. To minimize the uncontrolled BH_4 oxidation, due to environmental oxygen, an antioxidant, dithiothreitol (DTT), was used. The acidity of the medium, as well as the presence of hydrogen peroxide, affects the rate of the photoreaction and the nature of the obtained fluorescent photoproducts. The best conditions were achieved by irradiation in hydrochloric acid (0.2 M) medium, in presence of 100 mM hydrogen peroxide, and using an irradiation time of 20 min. The method was tested in the analysis of serum samples containing BH_4, and recoveries between 89 and 105% were found. Also, the proposed method allows the resolution of BH_4 and BIO, in the same sample, by injection of non-irradiated and irradiated sample aliquots in the chromatographic system.

1. Introduction

In humans, tetrahydrobiopterin (BH_4) has been recognized as the most important nonconjugated pteridin. BH_4 is a cofactor of aromatic amino acid hydrosylases involved in hydroxylation reactions of phenylalanine, tyrosine and tryptophan. The participation of BH_4 in neurotransmitter metabolism regulation allows knowing some type of phenylketonuria [1]. BH_4 is linked to its ability to reduce molecular oxygen generating electrons, transformation in 4-α-hydroxytetrahydrobiopterin and then, by dehydration, in quinonoid dihydrobiopterin (qBH_2) [2]. As a consequence, BH_4 is, at least, an essential cofactor of five enzymatic reactions; for phenylalanine-4-hydroxylase (PAH), tyrosine-3-hydroxylase, and tryptophan-5-hydroxylase. It is evident its implication in the phenylalanine—tyrosine transformation (Scheme 1). The alteration of the mentioned pathway gives rise to different types of hyperphenylalaninemia [3]. An amount of phenylalanine >150 μmol/L, in human serum, is considered as pathologic. Both, patients with defects in the biosynthesis of BH_4, as well as patients with BH_4 responsive phenylalanine hydrosylase (PAH) deficiency, benefit from substitution with the synthetic cofactor. BH_4 is administered orally at doses of 2–10 mg/kg body weight, in order to keep the normal hydroxylation of phenylalanine to tyrosine in the liver.

In addition to the hydroxylation of aromatic amino acids, BH_4 serves as the cofactor for nitric oxide synthase and glyceryl-ether monooxygenase. Nitric oxide (NO) is an important signalling molecule for vascular homeostasis by the regulation of blood vessel diameter, platelet aggregation, leukocyte adhesion and smooth muscle proliferation. It is believed that NO production is reduced by decreasing BH_4 concentration during oxidative stress, since it is one of the most potent naturally occurring reducing agents [4].

The main metabolite of BH_4 is biopterin (BIO). The determination of the neopterin/biopterin (NEO/BIO) ratio in urine, along with others analytical parameters, such as phenylalanine and tyrosine content, enzymatic activities evaluation and others, are applied to establish the differential diagnostic of the hyperphenylalaninemia.

Mother's milk contains high amounts of BIO, 90-fold than in serum, that fact indicates the possibility of production of BH_4 from mammary glands [1]. A number of data point out feeble permeability of cell membrane for BH_4; blood–brain barrier seems to be relatively impermeable for BH_4, which has been documented in animal experimental models and by mild efficacy in supplementation treatment with BH_4, in patients with deficiency in this compound [5]. Chemical reduction of biopterin yields two diastereoisomers, 6*R*- and 6*S*-BH_4, being 6*R*-BH_4 the natural form of tetrahydrobiopterin. Other important pteridine ring compounds are neopterin and biopterin [6].

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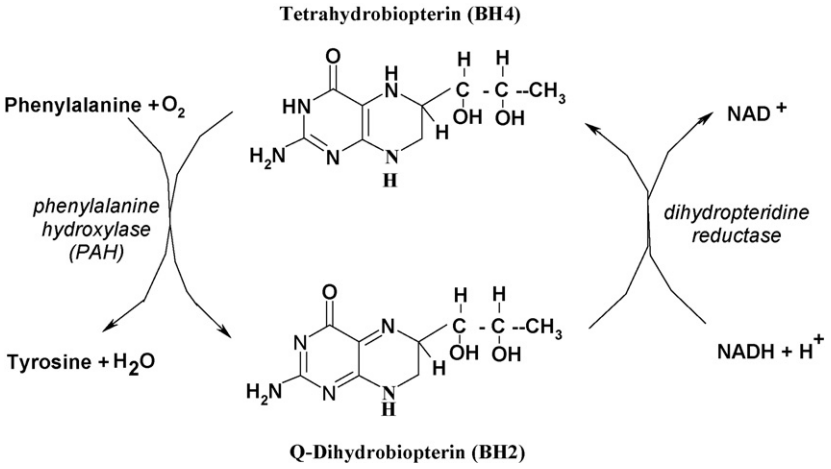


Scheme 1. Regeneration of 5,6,7,8-tetrahydrobiopterin (BH_4).

Most of the studies about the determination of BH_4 levels in plasma or urine are based on its indirect determination, by generation of biopterin, being the Fukushima method [7] widely applied. Briefly, the method consists on two steps: (1) by acid iodine oxidation the 7,8-dihydrobiopterin and BH_4 give rise to biopterin; (2) by alkaline iodine oxidation BH_4 gives rise to pterin and BH_2 undergoes to biopterin. The content of BH_4 in the original sample is calculated by difference between the amounts of biopterin obtained by acid and alkaline oxidation. It is described that the instability of BH_4 generates low content and poor repeatability in these analysis. Recently, two antioxidants have been tested, dithioerythriol (DTE) [8] and dithiothreitol (DTT) [9], in plasma. However, the presence of antioxidants could interfere in the later iodine oxidation [9].

Research studies about the photochemistry of folates, and other biologically active derivatives of pterin, indicate that the activity of the pterin heterocycle gives rise to changes of reduction degree of the original compounds. It is known that the attachment of formyl, methyl, methenyl or other one-carbon moieties to tetrahydrofolic acid affects the properties of excited molecules of folates. It is also known that photolysis of folic acid, folate derivatives and simple (non-conjugated) pterins, involves chemical bonds in the pteridine heterocycle and other portions of the molecule [10].

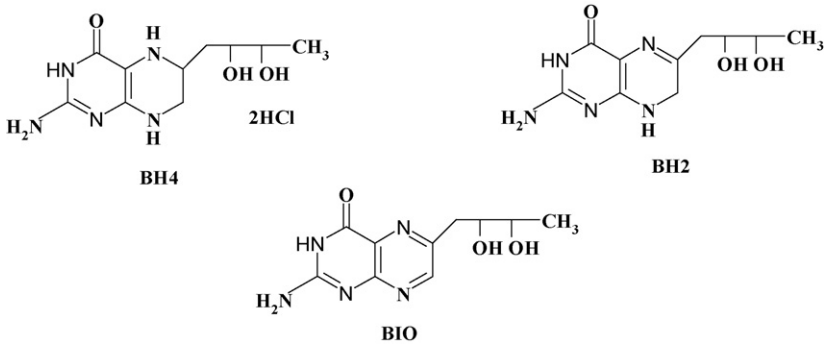
In previous papers, it has been observed how the photoirradiaton of some folates as folic acid, folinic acid and 5-methyltetrahydrofolic acid [11], is strongly affected by the UV irradiation. In some cases, a notable increment of the fluorescence is observed, and the generation of the corresponding oxidized pteridin appears feasible according to the spectral characteristics of the generated photoproducts. In other cases, the fluorescence disappears because a total oxidation of the molecule occurs. However, several photoproducts, with similar fluorescent characteristics, may be formed from the parent reduced forms.

With the aim of establishing the transformation in the oxidized pteridines, as an alternative way to the chemical oxidation described before, we have carried out several photoirradiation experiments accomplished with LC studies of some pteridin reduced forms. The 5,6,7,8-tetrahydrobiopterin (BH_4) and 7,8-dihydrobiopterin (BH_2) (Scheme 2) were studied as interesting biochemistry and clinical compounds. In both cases, the formation of biopterin would be desirable due to its high fluorescence quantum yield.

2. Experimental

2.1. Reagents

Pterin derivatives, as well as tetrahydro- and dihydro-derivatives, were purchased from Schircks Laboratories (Jona, Switzerland). BH_4, as dichorhydrate, was acquired as 6*R*-BH_4. All other chemicals were purchased from Sigma–Aldrich (Madrid, Spain). Stock standard solutions (90 $\mu g\,mL^{-1}$) of 5,6,7,8-tetrahydro-L-biopterin dihydrochloride (BH_4) and 7,8-dihydro-L-biopterin (BH_2), containing 0.1% DTT, were prepared by dissolution with ultrapure water. Exposure to direct sunlight was avoided.



Scheme 2. Chemical structures of the analytes, BH_4, BH_2 and BIO.