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Review

High-performance liquid chromatographic methods for the quantification of tetrahydrobiopterin biosynthetic enzymes

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Abstract

Tetrahydrobiopterin is a cofactor in hydroxylation reactions, including phenylalanine 4-monooxygenase, tyrosine 3-monooxygenase, tryptophan 5-monooxygenase, alkyl glycol ether monooxygenase and nitric oxide synthase. Determination of its biosynthesis is carried out to diagnose inherited diseases leading to partial defects in tetrahydrobiopterin synthesis. In addition, tetrahydrobiopterin synthesis is induced by proinflammatory cytokines, and intracellular levels of tetrahydrobiopterin in many cases limit the activity of tetrahydrobiopterin-dependent reactions, such as nitric oxide synthase in intact cells. Biosynthesis of tetrahydrobiopterin from guanosine 5′-triphosphate (GTP) requires the action of three enzymes, GTP-cyclohydrolase I (E.C. 3.5.4.16), 6-pyruvoyl tetrahydropterin synthase (E.C. 4.6.1.10) and sepiapterin reductase (E.C. 1.1.1.153). Methods for quantification of biopterin and related pteridines in biological matrices by HPLC and application of these for determining the activity of the three tetrahydrobiopterin biosynthetic enzymes are reviewed in this article.

Keywords: Reviews; Enzymes; Tetrahydrobiopterin

Contents

1. Introduction

Pteridines are a class of compounds with a common heterocyclic structure, the pyrazino-[2,3-D]-pyrimidine. Mammals have lost the ability to synthesize the pteridines riboflavin and folic acid, which are two vitamins. Other pteridines like molybdopterin, neopterin and biopterin, however, are synthesized by mammals (including humans) [1]. This review focuses on HPLC-based assays of enzymes

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carrying out the biosynthesis of tetrahydrobiopterin, a cofactor of phenylalanine 4-monooxygenase, tyrosine 3-monooxygenase, tryptophan 5-monooxygenase, alkyl glycol ether monooxygenase and nitric oxide synthase [2].

Biosynthesis of tetrahydrobiopterin has received attention for two major reasons: Rare inherited diseases leading to partially defective biosynthesis or recycling of tetrahydrobiopterin have been found [3]. These partial defects lead to high levels of phenylalanine and low levels of neurotransmitters such as dopamine, due to insufficient tetrahydrobiopterin cofactor, and hence insufficient activity of phenylalanine monooxygenase and probably some, if not all, of the other tetrahydrobiopterin-dependent enzymes. The second major reason is the stimulation of tetrahydrobiopterin biosynthesis by proinflammatory cytokines, which is crucial for optimal activity of nitric oxide synthase in cells (reviewed in [4]). Nitric oxide thus formed may serve a surpisingly heterogenous spectrum of roles, including cytotoxicity [5], control of parasitic infections [6] or increasing levels of cGMP [7], which in the blood vessel wall mediates relaxation of smooth muscles. Several other intriguing actions of tetrahydrobiopterin, such as control of cell cycle [8,9] or regulation of melanogenesis [10], are known.

Biosynthesis of tetrahydrobiopterin from guanosine 3′ triphosphate (GTP) requires three enzyme activities (Fig. 1): GTP-cyclohydrolase I (E.C. 3.5.4.16), 6-pyruvoyl tetrahydropterin synthase (E.C. 4.6.1.10) and sepiapterin reductase (E.C. 1.1.1.153). Since the first two of these enzyme activities, GTP-cyclohydrolase I and 6-pyruvoyl tetrahydropterin synthase are rather low in some tissues and cells (<1 pmol mg^{-1} min^{-1}), the sensitivity and resolution power of HPLC proved a valuable tool for the determination of these enzymes' activities in samples as complex as tissue- or cell-homogenates. Assay of GTP cyclohydrolase I and sepiapterin reductase can be done with commercially available reagents, whereas the assay of 6-pyruvoyl tetrahydropterin synthase requires GTP-cyclohydrolase I to prepare the substrate, 7,8-dihydroneopterin triphosphate, and, in the common protocols used, sepiapterin reductase to convert the unstable product that is formed into tetrahydrobiopterin.

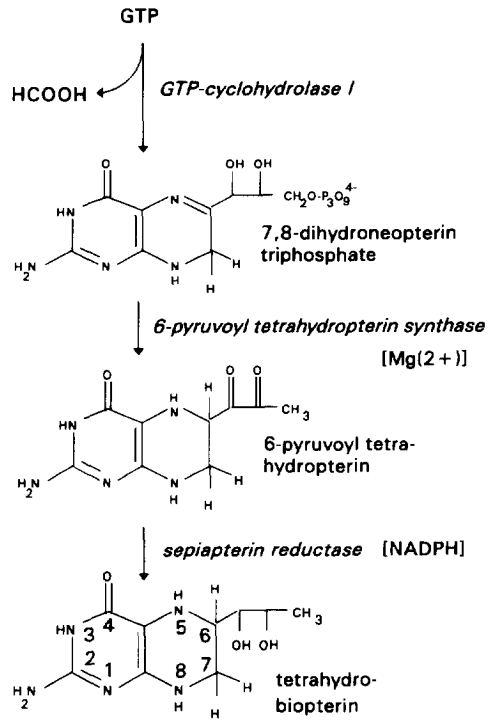

Fig. 1. Biosynthesis of tetrahydrobiopterin from GTP. GTP-cyclohydrolase I requires no additional cofactors, whereas Mg^{2+} is essential for 6-pyruvoyl tetrahydropterin synthase and NADPH is required by sepiapterin reductase to carry out their respective enzymatic reactions.

1.1. Determination of biopterin and related pteridines in biological samples by HPLC

1.1.1. Detection methods

Pteridines occur in urine in micromolar concentrations. In other body fluids as well as in tissue- and cell-extracts, concentrations of a few nmol/l are typical. Fortunately, the properties of pteridines allow sensitive and relatively specific detection of these compounds. One of these properties is the intense fluorescence, which is only observed when both rings are in the fully oxidised, aromatic state (e.g. neopterin in Fig. 2). The naturally occurring intermediates of tetrahydrobiopterin biosynthesis, 7,8-dihydroneopterin and 6-pyruvoyl tetrahydropterin, as well as the end-product, tetrahydrobiopterin, show no intense fluorescence. Due to their reduction potential, however, the reduced pteridines

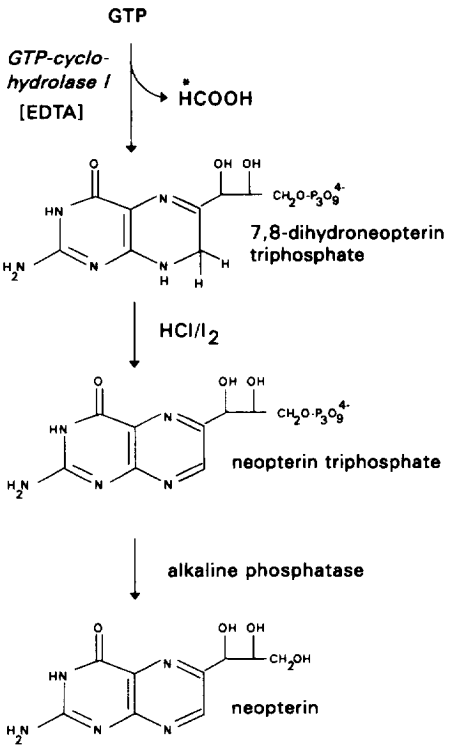

Fig. 2. Assays for GTP-cyclohydrolase I activity. A radiometric assay uses release of labelled formic acid from radiolabelled GTP. HPLC-based assays measure either neopterin triphosphate or neopterin using fluorescence detection.

may be monitored by electrochemical detection [11,12]. In contrast, defined oxidation of reduced, non-fluorescent biopterin derivatives to biopterin allows the use of the more robust fluorescence detection. If biopterin concentrations following oxidation in acidic solutions (which converts both 7,8-dihydrobiopterin and tetrahydrobiopterin to biopterin) and in alkaline solutions (which converts only 7,8-dihydrobiopterin to biopterin) are compared, the amount of the catalytically active tetrahydroform of biopterin can be estimated [13]. Alternatively, the use of sequential electrochemical and fluorescence detection [14,15] with coulometric electrodes carrying out quantitative redox-reactions in detection allows the determination of tetrahydro, dihydro and fully oxidised species in one run. The "quinonoid" 6,7[8H]dihydrobiopterin, the product of the enzymatic oxidation of tetrahydrobiopterin, can be monitored in this system by comparing results of analysis of samples without and with bisulfite, which traps the "quinonoid" 6,7[8H]dihydrobiopterin as an adduct and thereby alters its redox properties [16]. An elegant way to separate the different reduced froms of pteridines and combine this with the sensitivity and ease of fluorescence detection is post-column oxidation with nitrite [17]. GC–MS has occasionally been used to identify or confirm assignment of peaks in HPLC chromatograms [18–20] and one study employed LC–MS for the analysis of pteridines [21].

Electrochemical detection is difficult to perform in complex biological mixtures, and its application therefore focused mainly on cerebrospinal fluid, a relatively pure matrix. Application of the method to plasma samples requires removal of proteins by ultrafiltration [22]. In complex matrices, like tissue extracts or serum, we found that fluorescence detection allows a more robust routine analysis with less troubleshooting required.

1.1.2. Extraction and separation methods

For concentration of samples prior to HPLC analysis, Dowex resin columns [13,23], and solid-phase cartridges with [24], or without [25,26], direct insertion of cartridges into the HPLC-eluent stream, have been used. To remove protein from serum or tissue extracts, precipitation by acid [13,27], ultrafiltration [22,28] and deproteinization on a first column using column switching [29] were employed.

Separation systems commonly used include cation-exchange chromatography [30], reversed-phase chromatography [13,30–33], and ion-pair chromatography [14,15,17,29,30,34]. Addition of Cu^{2+} and D-phenylalanine to the mobile phase of reversed-phase HPLC allowed the separation of enantiomers of chiral pteridines [35].

Our impression is that ion-exchange and ion-pair chromatography approaches appear useful when different oxidation states of pteridines, which go along with different basicity of nitrogens 5 and 8, are to be separated. Also, separation of different tetrahydropterins [30], or the separation of mono-, di- and triphosphates [34], are readily performed on ion-pair or ion-exchange columns. If fully oxidised pterins with subtle differences in their side chains are to be

separated, however, reversed-phase appears advantageous. For the simultaneous determination of biogenic amines and pterins, a method using column switching has been designed [36]. The mobile phases employed for reversed-phase chromatography show subtle differences in the kind and amount of organic modifier used, and optimization of the mobile phase has been presented [37]. We have the impression that these slightly different mobile phases are specially adapted to the specific type of reversed-phase column used, which also slightly differ in their properties.

For protein removal, ultrafiltration appears to be the best method, since this leaves the redox state intact and imposes no restraints on the following procedures. Due to its simplicity it is more suited to routine application than the other methods. A drawback of ultrafiltration is the cost of the ultrafilters, which may only be used once. Acid precipitation is straightforward and shows results comparable to ultrafiltration. The high amount of acid, however, sometimes interferes with direct injection of the acidic reaction mixtures. Acidification also supports oxidation processes in some instances, so it is not suited for protein removal in cases where reduced pterins are to be monitored, unless chemical oxidation processes are carried out before, or together with, this step [13].

1.2. Assay of GTP cyclohydrolase I

Characterization of this enzyme from *E. coli* was done using an assay based on the release of radiolabelled formic acid originating from the labelled hydrogen atom in position 8 of GTP and separation of formic acid from GTP by charcoal [38]. Determination of GTP-cyclohydrolase I activity in mammalian tissues proved the power of HPLC for GTP-cyclohydrolase I measurements [39]. HPLC is superior to the radiometric method in that it measures the formation of the product. Degradation of GTP to products other than 7,8-dihydroneopterin triphosphate, however, may also lead to loss of label (for review see [40]).

The principle of GTP-cyclohydrolase I assays is shown in Fig. 2. GTP-cyclohydrolase I-containing tissue is first freed from low-molecular-mass compounds by molecular size exclusion chromatography on a short column (e.g. Sephadex G-25), incubated with excess GTP (1–2 m*M*) in the presence of EDTA for up to 90 min at 37°C. The presence of EDTA ensures that the product, 7,8-dihydroneopterin triphosphate, is not further metabolized, since the downstream 6-pyruvoyl tetrahydropterin synthase requires Mg^{2+} to operate [41]. The reaction is stopped by the addition of a mixture of HCl and iodine (dissolved in KI), which also oxidizes the labile 7,8-dihydroneopterin triphosphate to the somewhat more stable neopterin triphosphate. After completion of the oxidation, excess iodine is destroyed by adding ascorbic acid. Neopterin triphosphate may then either be analysed directly by ion-pair HPLC with fluorescence detection [34], or the mixture rendered slightly alkaline with NaOH and the triphosphate group cleaved off by treating with alkaline phosphatase to yield neopterin, which is analysed by reversed-phase HPLC with fluorescence detection [39]. The methods of Blau and Niederwieser [34] and of Viveros et al. [39], which were initially developed for use with mammalian tissues, also allowed GTP-cyclohydrolase I determinations to be carried out in cultured cells [42–44]. A modification of the method by Viveros et al. [39] uses accumulation of the formed neopterin by solid-phase extraction on strong cation exchanger cartridges and direct insertion of these cartridges into the stream of reversed-phase HPLC [24], which has been used to determine the induction of GTP-cyclohydrolase I activity in a variety of cultured cells [45].

Measurement of neopterin triphosphate avoids one incubation step and allows one to control for the presence of sufficient GTP at the end of the incubation time [34]. However, the stability of the triphosphate is limited even when purified GTP-cyclohydrolase I is used [46,47], due to chemical instability of the phosphate group. Measuring GTP-cyclohydrolase I in complex biological samples such as tissue- or cell-extracts will decrease the stability of the phosphates even more, due to the presence of phosphatases, which, in our experience, are not totally inhibited by the EDTA present in the incubation buffer. For these reasons, we recommend cleavage of the phosphates and measurement of neopterin by reversed-phase HPLC for determinations of GTP-cyclohydrolase I activity.

We found that the following experimental points

were crucial for successful GTP-cyclohydrolase I assays in tissue- and cell-extracts: Use of highly pure phosphatase is essential, since some phosphatase preparations contain GTP-cyclohydrolase I activity. Commercially available GTP may be contaminated to a variable extent with reduced neopterin phosphates, which interfere with the determination of low activities. Reagent blanks should therefore be run before using precious material. When cells are opened in the presence of antioxidants such as dithioerythrol, the amount of iodine must be increased accordingly. GTP-cyclohydrolase I is unstable in dilute solutions, thus a protein concentration of about 0.5 mg/ml should not be underscored. A detailed description of the protocol we are currently using will be published elsewhere [48].

1.3. Assay of 6-pyruvoyl tetrahydropterin synthase

Fig. 3 shows the enzymatic and chemical conversions employed for determination of 6-pyruvoyl tetrahydropterin synthase activities. An assay using the loss of a tritium label from the substrate has been described [49], employing adherence of the unconverted substrate to charcoal. Advantages of this method are its simplicity and sensitivity. The preparation of the labile, labelled substrate, however, although excellently worked out and well described [50], requires considerable effort. In addition, as with the GTP-cyclohydrolase I label release assay, it cannot be excluded *a priori* that reactions other than formation of the product 6-pyruvoyl tetrahydropterin may also lead to loss of the label. For applications where the most commonly used coupled assays (see below) cannot be employed, such as kinetic analyses, 6-pyruvoyl tetrahydropterin may be converted to pterin using iodine in acidic solution. Pterin can then be quantified by reversed-phase HPLC [55].

Most HPLC-assays of 6-pyruvoyl tetrahydropterin synthase activity, in contrast, measure formation of the product in an indirect way, since 6-pyruvoyl tetrahydropterin is labile and thus far not available as a synthetic compound. Therefore, further conversion to tetrahydrobiopterin using sepiapterin reductase [41], or conversion to 6-lactoyl tetrahydropterin using 6-pyruvoyl tetrahydropterin reductase [51], have been used. The tetrahydrobiopterin formed after reduction with sepiapterin reductase may be monitored electrochemically [41], or quantified after oxidation to biopterin by fluorescence detection [52–54]. 6-Lactoyl tetrahydropterin formed from 6-pyruvoyl tetrahydropterin is unstable in an aerobic environment and is converted spontaneously to sepiapterin, which can then be quantified by reversed-phase HPLC with UV/Vis detection at 420 nm. Compared to fluorescence and electrochemical detection, however, UV/Vis absorption has the drawback of lower sensitivity. In addition, this method

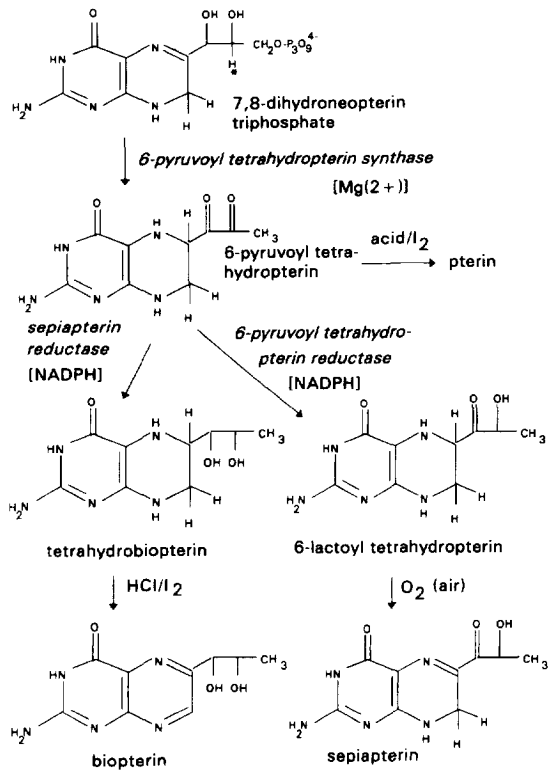

Fig. 3. Assays for 6-pyruvoyl tetrahydropterin synthase. The labile product of the reaction is either converted to tetrahydrobiopterin by sepiapterin reductase, which may be measured by HPLC using electrochemical detection. Alternatively, 6-pyruvoyl tetrahydropterin may be converted to pterin by oxidation with iodine in acidic solutions, and the resulting pterin quantified by reversed-phase HPLC with fluorescence detection. For detection of the activity in human cells, tetrahydrobiopterin is oxidized to biopterin, which is then quantified by HPLC with fluorescence detection, using column switching or solid-phase extraction techniques to separate the nanomolar amounts of biopterin from the 100 μM neopterin phosphates derived from 7,8-dihydroneopterin triphosphate, the substrate of the 6-pyruvoyl tetrahydropterin synthase reaction.

can only be used in the absence of sepiapterin reductase activities. Formation of pterin from 6-pyruvoyl tetrahydropterin is straightforward, but allows less sensitive quantification than obtained using the assay with fluorescent biopterin. A difficulty with this method is that the extent of the chemical conversion of 6-pyruvoyl tetrahydropterin to pterin cannot be readily determined in every assay.

A problem of the application of the 6-pyruvoyl tetrahydropterin synthase assays arises particularly with human tissues, which show up to two orders of magnitude lower activity than found in tissue- and cell-extracts of other mammals (e.g. rodents) [43,56,57]. Since the K_m of 6-pyruvoyl tetrahydropterin synthase for its substrate is 10 μM and since the activity of this enzyme may be <0.1 pmol mg^{-1} min^{-1}, e.g. in macrophages, the HPLC quantification of the incubation mixtures must be able to detect nanomolar concentrations of tetrahydrobiopterin in the presence of 100 μM of 7,8-dihydroneopterin triphosphate, which corresponds to ten times the K_m and is just sufficient to saturate the enzyme. Electrochemical detection can distinguish tetrahydrobiopterin from the dihydroneopterin triphosphate of the substrate, but appears only suited for enzyme purifications where ample activity occurs. Oxidation of tetrahydrobiopterin to biopterin, to take advantage of fluorescence detection, also converts the high excess of substrate to the fluorescent neopterin phosphates. These can be separated from biopterin either using column switching techniques [52,53], or extraction of the acidic reaction mixture with strong cation exchanger solid-phase columns [54], which do not retain the negatively charged neopterin phosphates, but retain (at acidic pH) the positively charged biopterin. An additional advantage of this extraction is the possibility of enriching the analyte, biopterin, from comparatively large (up to 1 ml) volumes of incubation mixture. This feature enabled detection of activity of 6-pyruvoyl tetrahydropterin synthase in human macrophages [58], which were found to be below the detection limit of previous investigations [59].

In addition to these analytical issues, critical points of 6-pyruvoyl tetrahydropterin synthase assays are the preparation of the labile substrate 7,8-dihydroneopterin triphosphate using purified GTP-cyclohydrolase I, as well as the preparation of sepiapterin reductase that is pure enough to be free of residual 6-pyruvoyl tetrahydropterin synthase activity. Availability of the enzymes in recombinant form [60–62] greatly facilitates the purification of these enzymes by single column procedures. For the preparation of 7,8-dihydroneopterin triphosphate, repetitive recycling of GTP-cyclohydrolase I has been employed [63]. However, our best results were obtained by freshly preparing dihydroneopterin triphosphate just before the assay and adding the formed dihydroneopterin triphosphate together with the GTP-cyclohydrolase I to the incubation mixture [54].

1.4. Sepiapterin reductase assay

Compared to the two aforementioned enzymes, GTP-cyclohydrolase I and 6-pyruvoyl tetrahydropterin synthase, sepiapterin reductase has two to three orders of magnitude higher activity in human and in other mammalian cells. Typical activities are found in the order of magnitude of 500 pmol mg^{-1} min^{-1} [44,58,59,64,65]. Therefore, sepiapterin reductase is easily assayed in a comparatively small amount of cells, using the procedure outlined in Fig. 4 [64]. Although 6-pyruvoyl tetrahydropterin rather than sepiapterin is the substrate of sepiapterin reductase in tetrahydrobiopterin biosynthesis (see Fig. 1), the activity of this enzyme is commonly assayed using the more stable, artificial substrate, sepiapterin. In the presence of dihydrofolate reductase activities, 7,8-dihydrobiopterin will be further converted to tetrahydrobiopterin during the incubation step. The oxidation in acidic media also will convert this product to the fluorescent biopterin, so that no alteration of the result arises from the additional presence of dihydrofolate reductase.

2. List of abbreviations

7,8-Dihydroneopterin triphosphate	7,8-dihydro D-neopterin 3′-triphosphate
GTP	Guanosine 5′-triphosphate
Tetrahydrobiopterin	6*R* 5,6,7,8-tetrahydro L-biopterin

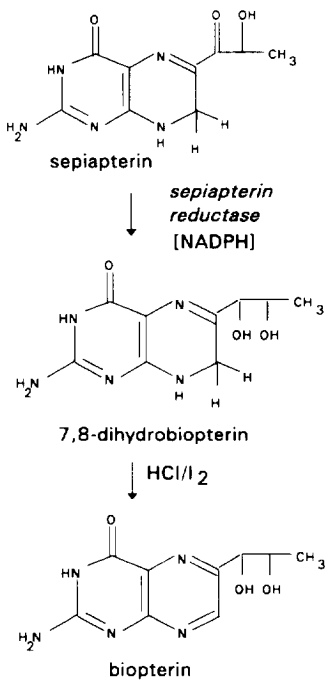

Fig. 4. Assay for sepiapterin reductase. 7,8-Dihydrobiopterin, formed from sepiapterin, is oxidized to biopterin in acidic media and measured by HPLC with fluorescence detection. Compared to GTP-cyclohydrolase I and 6-pyruvoyl tetrahydropterin synthase, human cells contain two to three orders of magnitude higher activities of sepiapterin reductase, so that this activity is easily measured without approximation of the sensitivity limit of the detection system.

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