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REVIEW

ANALYSIS AND CLINICAL SIGNIFICANCE OF PTERINS

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LIST OF ABBREVIATIONS

CSF	Cerebrospinal fluid
DETAPAC	Diethylenetriaminepentaacetic acid
BH2	Dihydrobiopterin
DHPR	Dihydropteridine reductase
DTE	Dithioerythritol
GTP	Guanosine triphosphate
GC–MS	Gas chromatography–mass spectrometry
HPLC	High-performance liquid chromatography
q-BH2	Quinonoid dihydrobiopterin
RIA	Radioimmunoassay
BH4	6*R*-L-*erythro*-5,6,7,8-Tetrahydrobiopterin
TLC	Thin-layer chromatography

1. INTRODUCTION

Although the history of pterins began in 1889 with the study of the yellow pigment found in butterfly wings [1] it was not until 1940 that the true structure of a member of this group of compounds (xanthopterin) was elucidated [2]. Since then three crucial discoveries have led to a rapid increase of interest in these compounds: (1) the discovery of the requirement of tetrahydrobiopterin (BH4) as a cofactor for the aromatic amino acid hydroxylases [3–8]; (2) the recognition that inborn errors of the metabolism of BH4 could lead to hyperphenylalaninaemia [9]; and (3) the finding of a change in urinary pterin patterns, especially neopterin, in humans with malignant and infectious diseases [10]. It was the need to have a better understanding of the mechanism involved in these processes which lead to the development of methodology which may be used in the clinical laboratory for the analysis of pterins.

1.1. Structure of pterins

Pterins are members of a group of compounds known as pteridines. They have a nucleus of a 2-amino-4-hydroxypteridine (pterin) (Fig. 1) but differ in the nature of the side-chain at position 6 and the state of oxidation of the ring which may exist in the tetrahydro, dihydro or a fully oxidised form. The biologically

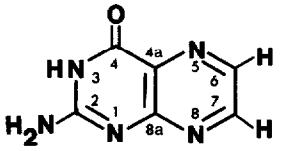


Fig. 1. Structure of pterins.

TABLE 1

PTERINS IDENTIFIED IN HUMAN TISSUES AND FLUIDS

Compound	Side-chain	Reference
Biopterin	C-6-CHOH-CHOH-CH_3	11
7,8-Dihydrobiopterin	C-6-CHOH-CHOH-CH_3	11
5,6,7,8-Tetrahydrobiopterin	C-6-CHOH-CHOH-CH_3	12
Neopterin	C-6-CHOH-CHOH-CH_2OH	11
7,8-Dihydroneopterin	C-6-CHOH-CHOH-CH_2OH	11
threo-Neopterin (monapterin)	C-6-CHOH-CHOH-CH_2OH	11
Sepiapterin	C-6-CO-CHOH-CH_3	11
3′-Hydroxysepiapterin	C-6-CO-CHOH-CH_3, C-3-OH	13
Isosepiapterin	C-7-CO-CHOH-CH_3	11
Xanthopterin	C-6-OH	11
7,8-Dihydroxanthopterin	C-6-OH	14
Isoxanthopterin	C-7-OH	15
Pterin	C-6-H	11
Pterin-6-aldehyde	C-6-CHO	16
6-Carboxypterin	C-6-COOH	11
6-Hydroxymethylpterin	C-6-CH_2OH	12
Molybdenum cofactor	See Fig. 4	18

relevant compounds containing this structure may be divided into two groups: (1) those containing a simple hydroxyalkyl group at position 6, which are known as pterins, and (2) those linked to *p*-aminobenzoylglutamates(s) which are referred to as folates. This review will be based only on the measurement and importance of pterins in clinical disease. A list of the compounds that can be defined as pterins that have been identified in man and the nature of the side-chain is given in Table 1. In many cases it is not known whether these compounds have any active biological function, or if they are produced enzymatically, or whether they are just chemical oxidation products of the biologically active pterins.

1.2. Biosynthesis of pterins

The well defined functions of pterins in man are limited to the involvement of *6R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH4) in the hydroxylation of phen-

ylalanine [19,20], tryptophan [5,7] and tyrosine [8], and the participation of molybdopterin as the cofactor for xanthine (EC 1.2.3.2) [21], sulphite (EC 1.8.3.1.) [22] and aldehyde oxidase (EC 1.2.3.1) [23]. The elucidation of the biosynthetic pathways of these two cofactors is still under investigation. Although a great deal is known about that leading to BH4, very little information is known regarding the biosynthesis of the molybdenum cofactor.

1.2.1. Tetrahydrobiopterin

The history of the elucidation of the pathway of BH4 synthesis has been reviewed elsewhere [24] and hence will not be described here. BH4 is synthesised from guanosine triphosphate (GTP) [25–27] via a series of reactions which are as yet not fully characterised (Fig. 2). The first, catalysed by GTP cyclohdrolase (EC 3.5.4.16), leads to the conversion of GTP to D-*erythro*-dihydroneopterin triphosphate with the loss of a formate group [28,29]. Subsequently dihydroneopterin triphosphate is converted to BH4 by at least two further enzymes via a series of tetrahydropterin intermediates [30–33]. The first step in this sequence is catalysed by a single enzyme, 6-pyruvoyl tetrahydropterin synthase, which catalyses the elimination of the triphosphate and an internal oxidoreduction [34]. The sequence of the two reactions is still undetermined. The 6-pyruvoyl tetrahydropterin formed then undergoes reduction of the two keto groups on the alkyl sidechain to yield BH4. There is still a question as to whether these two reductions are performed by sepiapterin reductase (EC 1.1.1.153) or whether another enzyme, 6-pyruvoyl tetrahydropterin reductase, is also required [31]. Whether the reduction occurs first at the C-1′ or at the C-2′ position is also unknown [31].

For BH4 to be functional in the hydroxylation of the aromatic amino acids another enzyme is necessary, which although not on the biosynthetic pathway, is required to regenerate BH4 following its oxidation. The enzyme, dihydropteridine reductase (DHPR) (EC 1.6.99.7), reduces the quinonoid dihydrobiopterin (q-BH2) formed during the hydroxylation back to BH4 in an NADH-dependent reaction (Fig. 3) [35].

1.2.2. Molybdenum cofactor

None of the enzymatic steps leading to the synthesis of the molybdenum cofactor are known in man. The proposed structure of the cofactor is shown in Fig. 4 [18]. The cofactor does not seem to be synthesised from GTP via the action of GTP cyclohdrolase as patients with a genetic deficiency of this enzyme do not show decreased activities of xanthine and sulphite oxidase [36].

1.3. The involvement of pterins in pathological processes

Abnormalities of the synthesis and metabolism of BH4 and the absence of the molybdenum cofactor lead to clinical problems in man.

1.3.1. Tetrahydrobiopterin deficiency

The role of BH4 as a cofactor for the aromatic amino acid hydroxylases [37] means that any condition leading to BH4 deficiency will result in:

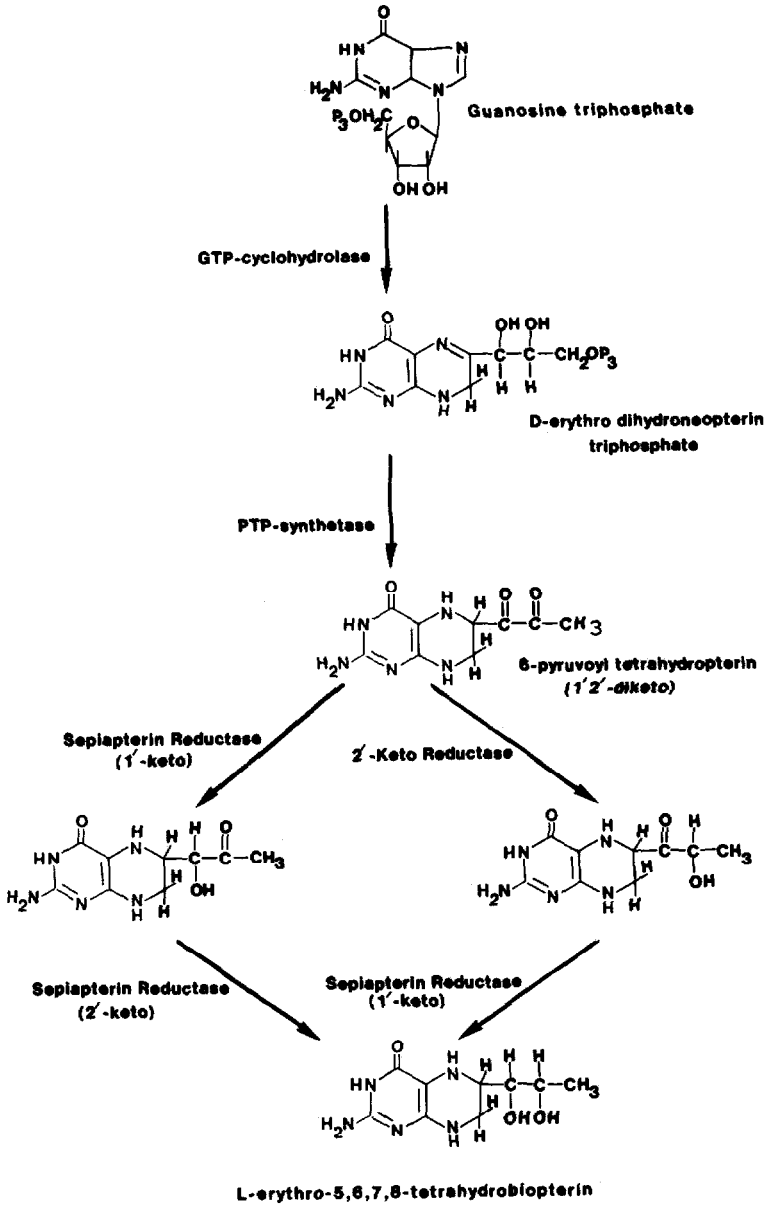


Fig. 2. Synthesis of tetrahydrobiopterin from guanosine triphosphate. It is still uncertain whether 6-pyruvoyltetrahydropterin is first reduced at the 1'-keto or the 2'-keto position. PTP-synthetase = pyruvoyltetrahydropterin synthetase.

(1) Hyperphenylalaninaemia due to decreased phenylalanine hydroxylation (Fig. 3).

(2) Deficiency of serotonin, as the conversion of tryptophan to 5-hydroxytryptophan, catalysed by tryptophan hydroxylase (EC 1.14.16.4), is the rate-limiting step in serotonin synthesis [37] (Fig. 3).

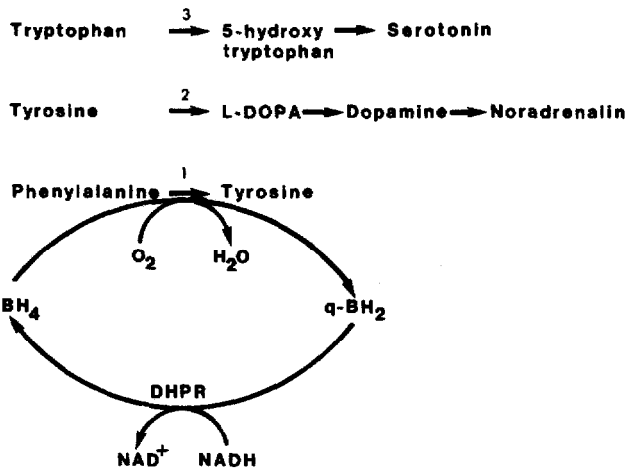


Fig. 3. Involvement of dihydropteridine reductase (DHPR) in the hydroxylation of the aromatic amino acids. 1 = Phenylalanine hydroxylase; 2 = tyrosine hydroxylase; 3 = tryptophan hydroxylase.

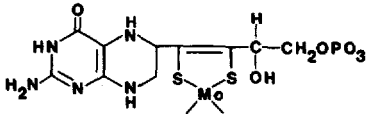
(3) Deficiency of the catecholamines as tyrosine hydroxylase (EC 1.14.16.2) is the rate-limiting step in dopamine synthesis [37] (Fig. 3).

Deficiency of BH4 may arise because of inborn errors of metabolism of the enzymes of the biosynthetic pathway [36,38,39] or of DHPR [9] or as a result of chemical interference of BH4 metabolism [40–42].

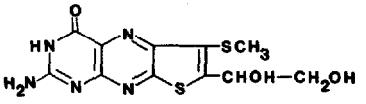
1.3.2. Molybdenum cofactor deficiency

The requirement of molybdopterin for the normal functioning of xanthine and sulphite oxidase means that deficiency of this pterin leads to a failure of oxidation of xanthine and S-sulfocysteine and their excretion in large quantities into the urine [43]. Deficiency of molybdopterin has only been demonstrated in patients with inborn errors of metabolism; it is not yet known whether chemical agents can affect the concentration of this cofactor.

The main urinary excretion product of molybdopterin is urothione (Fig. 4). Since this compound is absent in the urine from patients with molybdenum co-



Molybdenum cofactor



Urothione

factor deficiency, measurement of this compound has been used in the diagnosis of this condition [44,45].

As well as a deficiency of pterins causing pathological processes, many pathological situations themselves are accompanied by secondary changes in pterin metabolism. These changes can be used to gain information on the type, prognosis, course and success of the clinical management of the disease.

1.3.3. Pterins in immunology

In many situations where there is an alteration of the immune system status, e.g. cancer, viral infection, bacterial infection and pregnancy, there is an increase in the concentration of dihydro- and fully oxidised neopterin in serum [46], urine [47] and cerebrospinal fluid (CSF) [48].

The neopterins are released in vitro from macrophages [49] following γ-interferon stimulation of GTP synthesis and GTP cyclohydrolase activity within these cells [50]. It is probable that a similar mechanism acts in vivo.

The reason for the neopterin release is unclear. Patients with GTP cyclohydrolase deficiency show no signs of immuno deficiency and therefore it is unlikely that the neopterin plays an active role in the immune process [36]. A recent hypothesis has been proposed suggesting that oxidised neopterin may have an anti-folate activity by inhibiting folate metabolism in intracellular pathogenic microorganisms [51].

Although neopterin does not appear to have a vital role in the immune process, measurement of this pterin in biological fluids has proved useful for monitoring diseases where there is change in the immune system homeostasis (see ref. 47 for review).

1.3.4. Diverse neurological diseases

Disturbed pterin metabolism has been implicated in many neurological diseases. Decreased concentrations of BH4 have been reported in CSF from patients with Parkinsons disease [52–54], Shy Drager and Steele Richardson syndromes [55], dystonia [56], Alzheimers disease [57–59] and 5,10-methylenetetrahydrofolate reductase (EC 1.1.1.68) deficiency [60,61]. In all of these cases it is not clear whether a problem in BH4 metabolism is the primary defect or whether pterin metabolism is altered as a secondary event following dopaminergic or serotonergic neuronal loss.

BH4 metabolism may also be altered on chronic or acute exposure to lead [40,41], aluminium [42] or methotrexate [62] and it is possible that the altered pterin metabolism may contribute to some of the neurological signs associated with exposure to these agents.

2. ANALYSIS OF PTERINS

2.1. Collection and storage of clinical samples

When considering methods of sampling, storage and analysis of pterins, numerous factors need to be noted:

(1) Pterins exist in three oxidation states.

(2) Pterins are photosensitive.

(3) Reduced pterins are easily oxidised in the presence of air, especially if any metal ion contaminants are present [63,64].

(4) Reduced pterins do not always oxidise to their fully oxidised parent compound [e.g. BH4 and dihydrobiopterin (BH2) do not always oxidise to biopterin] [64–67].

(5) Loss of pterins due to oxidation will lead to serious underestimation due to conversion to undetected pterin forms and other non-pterin breakdown products [64–68].

The above points indicate that sample handling should be critical if reliable results are to be obtained. However, many established methods for measuring pterins in clinical samples do not appear to depend on perfect sample handling for the provision of clinically useful data. This leaves one to reflect on how much more information could be obtained if the samples were collected and stored under conditions where no loss or interconversion of pterins occurs.

The main clinical samples collected for pterin analysis are urine, whole blood, plasma or serum, and CSF. The literature regarding the stability of pterins in these samples is extremely sparse.

2.1.1. Urine

Many groups, using a variety of different analytical techniques, have described the measurement of either one particular pterin or a group of pterins in urine [11,12,14–16,38,69–92]. In many cases no mention is made of the method of collection or storage of the urines. Some state the need to collect the specimens in light-protected containers [12,74,76,83,84,86]. Others have added concentrated hydrochloric acid to urine in order to stabilise BH4 [70]; however, this is of little use if the urine has to be collected in a bag attached to children or older incontinent patients. It may also lead to substantial losses of dihydroneopterin as this pterin is unstable at acid pH.

In our hands [15] and those of others [85], the best method of protection of samples is by the voiding of urine into a container having ascorbic acid present to give a final concentration of 1–10 mg/ml. If the urine has to be taken from young children the ascorbic acid should be placed directly in the urine collection bag which should be removed and the contents transferred to a darkened container and frozen as quickly as possible. The pterins collected in this manner are stable for months when stored at between $-20°C$ and $-70°C$ [15]. We have, however, noticed that freezing and thawing can lead to considerable losses of dihydroneopterin which are not reflected by a quantitative increase in neopterin concentrations (Table 2). There is no interconversion or loss of biopterins following freezing and thawing.

Precipitate removal by centrifugation should be avoided at all costs if reduced pterins are of interest, as a large proportion of these associate with, and are removed with, the precipitate [14, unpublished observations].

Since there is no significant diurnal variation of pterin excretion [74], random urine samples can be used provided results are expressed in terms of urine cre-

TABLE 2

OXIDISED NEOPTERIN AND DIHYDRONEOPTERIN CONCENTRATIONS IN URINE FROM PATIENTS SUFFERING FROM CEREBRAL MALERIA: EFFECT OF FREEZING AND THAWING

Urine was passed directly into foil-wrapped vessels containing ascorbic acid to give a final concentration of approximately 1 mg/ml. Samples were placed on dry ice and then stored at −70°C until analysed. Samples were refrozen to −70°C after the first thawing.

Sample	Times of freezing and thawing	Oxidised neopterin (ng/ml)	Dihydroneopterin (ng/ml)
1	1	1207	4421
	2	2256	218
2	1	6454	8128
	2	8128	756
3	1	7486	11323
	2	12390	170
4	1	5025	13458
	2	8800	1078
5	1	4105	10437
	2	4992	2876
6	1	5988	20373
	2	7712	658

atinine concentrations. However, pterin excretion patterns change with age, and therefore aged-matched controls should be used especially in the first year of life [92].

2.1.2. Cerebrospinal fluid

To our knowledge we are the only group to have performed a detailed study of stability of pterins in CSF [67]. Our studies have demonstrated that reduced pterins in CSF are extremely labile and that their loss is not accompanied by a corresponding rise in oxidised pterins. Therefore incorrect collection, storage and processing of CSF samples can lead to serious underestimation of the pterins present.

These problems can be overcome by collecting CSF directly into tubes containing 1 mg/ml dithioerythritol (DTE) and 1 mg/ml diethylenetriaminepentaacetic acid (DETAPAC) and storing the samples at −70°C until analysed [48,67]. Samples collected and stored in this manner are stable for longer than one year and following thawing are stable for up to 2 h at room temperature. This allows sufficient time for any sample preparation that may be required.

2.1.3. Blood

As for the other body fluids, the information regarding the stability of pterins in venous blood is extremely limited. Whole blood dried on Guthrie cards (obtained from the Department of Health and Social Security in the United Kingdom, and used for the general screening for phenylketonuria and hyperthyroidism) gives consistent total "biopterin equivalent" concentrations as measured by the

Crithidia bioassay [93] (see Section 2.4.1.) but since it is unknown whether there is interconversion or loss of biopterins during storage or during elution prior to analysis, this technique of sample collection may not be suitable for more detailed analysis of pterins.

Oxidised neopterin concentrations in serum are relatively stable for up to five days when stored at 4°C; however, during this period there is an almost total loss of reduced neopterins which is not accompanied by a corresponding increase in oxidised neopterin [68]. Serum samples may be stored at −20°C without loss or interconversion of reduced or oxidised neopterin [68]. No information is available regarding the loss of biopterin during storage or of the loss of pterins during the separation of the serum.

Plasma analysis of pterins is also used for diagnostic purposes and here it is again assumed that there is no loss of pterins during separation of the plasma. We have conducted preliminary studies of the stability of BH4 in human blood and have found that the slightest haemolysis leads to a total loss of BH4 from plasma as measured by electrochemical detection following high-performance liquid chromatography (HPLC) (unpublished observations).

A detailed study of the stability of all pterins in biological fluids and the determination of the conditions which prevent alteration of pterin concentrations and interconversion of the different oxidation states during sample handling would undoubtedly make a significant contribution to the literature regarding the use of pterin measurement in the diagnosis and monitoring of human disease.

2.2. Preparation of samples

The procedures required for the preparation of samples before analysis vary depending on: (1) the type of analytical method, (2) the comprehensive nature of the analysis, i.e. is just one pterin or are many different pterins going to be measured, (3) the necessity to preserve the original oxidation states of the individual pterins during the analysis and (4) the complexity of the biological sample (blood, CSF, urine or tissue).

Due to the diversity of requirements and the multitude of sample preparation procedures (or in some cases their absence), which have been published by various groups using many analytical techniques, details of sample preparation will be included where relevant in the following section describing the methods themselves.

2.3. Chromatographic methods

2.3.1. High-performance liquid chromatography

Chromatographic analysis of pterins in the present-day clinical laboratory relies almost entirely on HPLC techniques, and most of the work involves the measurement of neopterins and biopterins in various diseases. These pterins have been separated using cation-exchange [70,74,94,95], reversed-phase [17,48,73,75,86,87,96] and reversed-phase ion-pair chromatography [12,15,97] with detection by UV [94] fluorescence [17,70,73–75, 84,86,87,89,95,96], electro-

chemistry [12,97] and electrochemistry in conjunction with fluorescence [15,48,67]. Many of the above methods are also able to separate and quantify pterins other than neopterin and biopterin.

2.3.1.1. Fluorescence detection. Fluorescence detection is used extensively as it has the necessary sensitivity and selectivity for the measurement of pterins in biological samples. A problem does, however, exist as it is only oxidised pterins which fluoresce, and most, if not all pterins, are present endogenously in their reduced forms [85]. Reduced pterins must therefore be oxidised chemically prior to analysis.

Using a technique whereby pterins are differentially oxidised by iodine under acid and alkaline conditions, all three oxidation states of biopterin can be measured [73,98,99]. This oxidation procedure can be used for all tissues and fluids and relies on the following principles:

(1) Fully oxidised biopterin is unaffected by acid or alkaline iodine oxidation.

(2) BH2 and BH4 are both quantitatively converted to biopterin by iodine under acid conditions.

(3) BH2 is quantitatively converted to biopterin by iodine under alkaline conditions, whereas BH4 is converted to pterin.

Hence after sample purification and HPLC separation, endogenous biopterin can be measured following direct injection of sample without prior oxidation. Total biopterin (biopterin + BH2 + BH4) concentrations can be measured by injection of the acid-oxidised sample. BH2 concentrations can be estimated by subtraction of the endogenous biopterin from the total found after alkaline oxidation, and BH4 by subtracting the biopterin concentration found following alkaline oxidation from that found following acid oxidation. The principles of this method are summarised in Table 3.

The main advantage of the oxidation procedure lies in the fact that the labile reduced pterins are rapidly converted to the stable oxidised forms. This removes the possibility of loss or interconversion of reduced pterins if subsequent clean-up steps are required prior to chromatography. The method has a major disad-

TABLE 3

PRINCIPLES OF THE DIFFERENTIAL ACID AND ALKALI IODINE OXIDATION FOR THE ANALYSIS OF TETRAHYDRO-, DIHYDRO- AND OXIDISED PTERINS

Full details of the method are described in ref. 73.

Iodine oxidation conditions	Constituents of biopterin peak measured after HPLC
(1) Acid	B + BH2 + BH4
(2) Alkaline	B + BH2
(3) No oxidation	B
Therefore:	
endogenous biopterin	= (3)
BH4	= (1) − (2)
BH2	= (2) − (3)

vantage if one is studying the endogenous oxidation forms of neopterin, as dihydroneopterin is not reliably converted to neopterin under alkaline conditions [73]. Also there is still some question as to whether tetrahydroneopterin is found in human samples [12,15,91].

If measurement of each oxidation state of the pterins is not important and only a "total" value for each pterin is required, a simple manganese dioxide acid oxidation procedure has been developed [76,89] which is suitable for urine and CSF [76,89,100].

Some laboratories have experienced problems with this oxidation procedure which appear to be related to the type of mangenese dioxide used. If, however, a fine powder grade is used and the samples are kept in the dark and well agitated during the oxidation process, quantitative conversion of reduced pterins to their oxidised forms is achieved [89].

Following oxidation of reduced pterins by one of the above methods, sample clean-up prior to chromatography is generally achieved using a two-stage ion-exchange protocol. Briefly this involves addition of an internal standard (6-methylpterin [70], 6,7-dimethylpterin [84] or 6-hydroxymethylpterin [89]), application of the sample to a Dowex 50W-X8 (H^+, 200–400 mesh) column, washing with water (which should be collected if isoxanthopterin is to be measured [84]) and elution of pterins with ammonium hydroxide. The eluate is applied to a Dowex 1-X8 (OH^-, 200–400 mesh) column, washed with water, and the pterins eluted with acetic acid. Full details of this sample clean-up have been described [70,73,74].

Several methods have been developed for the measurement of oxidised pterin profiles in iodine- or manganese dioxide-treated urines which dispense with the need for the above sample preparation. These rely on gradient elution [86] or gradient elution together with column switching [89] to prevent contamination and loss of resolution of the analytical column. A simple isocratic system has also been described for direct urine injection [87] but no information has been given regarding column life.

Methods of direct urine injection have an added advantage in that creatinine concentrations may be measured during the same analytical analysis by placing a UV detector after the fluorimeter [86,87,90]. Creatinine is not affected by iodine oxidation [86].

As well as the above methods which are used to measure many different pterins, reversed-phase chromatographic systems have been described which are used to specifically measure oxidised neopterin in either urine [75,90] or serum [96]. Sample preparation is minimal (passage through a Sep-Pak C_{18} cartridge) [75] or absent [90] and again gradient elution [75] or column switching [90] is used to prevent contamination of the analytical column. Neopterin estimation in patients receiving salicylazosulfapyridine can be problematic as such patients excrete highly fluorescent byproducts [101].

2.3.1.2. Electrochemical detection. Fluorescence detection does not allow the direct measurement of reduced pterins, and the differential oxidation procedure requires the running of three chromatograms to achieve an "estimation" of reduced pterin concentrations. Electrochemical detection provides a method of di-

rectly measuring the endogenous oxidised and reduced pterins within biological samples.

Before embarking on the direct measurement of reduced pterins by HPLC it is important to recognise that tetrahydropterins are readily oxidised on the analytical column during the chromatographic analysis [15,48,94]. This is particularly apparent when concentrations of the reduced pterins are low as in CSF [48,67]. Techniques to prevent this oxidation have included displacement of oxygen from the mobile phase by bubbling with nitrogen [102] or argon [97], injection of small quantities of sodium metabisulphite prior to the analysis [94] or addition of antioxidant to the mobile phase [100]. From our own work we have found that the addition of DTE to the mobile phase completely prevents the oxidation of BH4 even at the low concentrations found in CSF [15,48,67].

The ease by which electrochemistry can be used to detect pterins depends on the state of oxidation of the molecule. The potential required to oxidise BH4 is very low. This allows the measurement of this pterin without any sample purification in urine [15,89,97] and CSF [48,67] and even tissues following removal of protein [103], providing adequate measures are taken to prevent oxidation before sample injection and during the chromatographic analysis. Original methods describing the measurement of BH4 used a single amperometric electrode [89,97] but this has a number of limitations. It is not possible with a single electrode to simultaneously measure dihydro- and tetrahydropterins in biological samples without prior sample clean-up, as the potential required to oxidise dihydropterins is much greater than that for the tetrahydropterins. The high potential results in the oxidation of many other compounds within the sample and the generation of extremely complex chromatograms. Furthermore a single electrode does not allow simultaneous detection of oxidised and reduced pterins as a reducing potential must be set on the electrode for detection of the oxidised molecules.

Two different methods have subsequently been described which allow simultaneous detection of reduced and oxidised pterins. A system having two amperometric electrodes in the parallel adjacent configuration has been described [102,104]. One electrode is set at a high oxidation potential for the detection of reduced pterins and the other is set at a high reducing potential for the detection of oxidised pterins. Because of the high potentials required sample pretreatment is necessary. Reduced pterins do not bind strongly to polystyrene-based resins and oxidised pterins are not sufficiently retained by Sephadex-type resins to afford a single-step procedure [102]. A two-step clean-up procedure using SP-Sephadex C_{25} and Dowex 50-X8 (H^+, 200–400 mesh) does give high, reproducible recoveries [102].

The design of the amperometric electrode only allows for oxidation or reduction of a small proportion of the total number of molecules passing through the detector. Another type of electrochemical electrode is available which effectively oxidises or reduces 99% of the molecules. This is achieved by passage of the sample through a porous graphite electrode to which the potential is applied. The high efficiency results from the large area of the electrode surface. This "coulometric" electrode has several advantages when used for pterin analysis.

BH4 has a low redox potential and therefore a low positive potential will oxidise the molecule and a low negative potential will reduce it back again. There are very few compounds in biological materials which can be oxidised and reduced at the low potentials which are used for BH4. By placing two coulometric electrodes in series, with a low oxidation potential on the first and a low reducing potential on the second, and measuring the BH4 at the second electrode, a very specific method is available for the measurement of BH4 (Fig. 5). Coulometric electrochemistry has been used for the direct measurement of BH4 in urine [15] and CSF [48,67], in either the single-cell oxidative [15] or dual-cell redox modes [48,67].

Redox detection of BH4 has another distinct advantage in that ascorbic acid [15,85] and DTE [48,100] (the two common antioxidants used to protect BH4 in urine, CSF and tissues during storage and sample preparation), although easily oxidised, are not reduced back again at the low negative potentials used for the measurement of tetrahydropterins. Therefore, although these antioxidants produce a large electrochemical signal on an oxidation chromatogram which can obscure the BH4 peak, especially if the BH4 concentration is low, no signal is seen from the reducing electrode (Fig. 6). High concentrations of the antioxidant may therefore be added to the sample at the time of collection ensuring no oxidation of the reduced pterins during storage and future sample handling.

The coulometric electrode also provides a means of detection of dihydropterins in biological samples in a manner which does not require extensive pre-purification of the material. Passage of dihydropterins through a coulometric cell set at a high oxidation potential leads to oxidation of the dihydropterin to the fully oxidised molecule [15]. These oxidised pterins are fluorescent and by placing a fluorescence detector in series after the electrochemical cell the dihydropterins may be quantified [15].

By placing three coulometric cells in series prior to a fluorescence detector all three oxidation states can be measured directly in urine and CSF without any

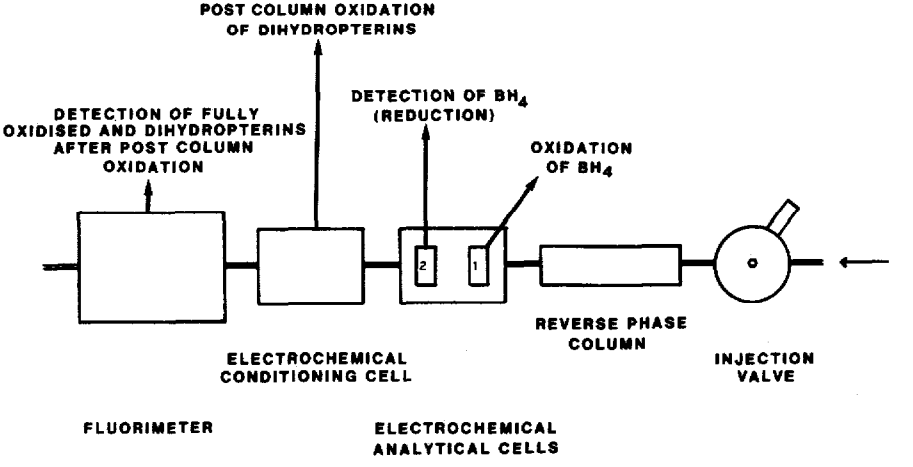


Fig. 5. Equipment configuration for the measurement of tetrahydro-, dihydro-, and fully oxidised pterins.

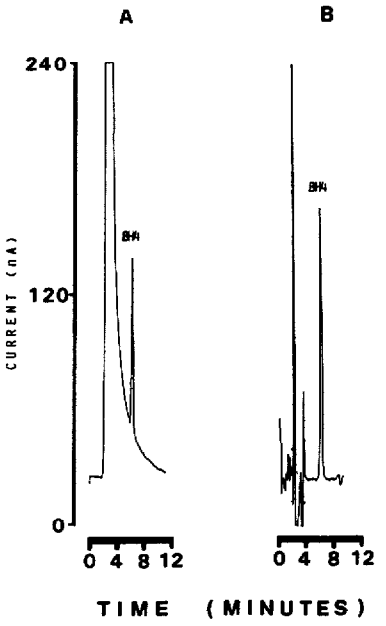


Fig. 6. Chromatogram of tetrahydrobiopterin (77.5 ng/ml) using electrochemical detection. (A) Detection with oxidising electrode only; (B) detection in the redox mode. Ascorbic acid was present at 1 mg/ml.

sample pre-purification [15,67,48] and in brain following acid precipitation of protein (unpublished observations). Thus: (1) tetrahydropterins are measured by electrochemistry using two coulometric electrodes set in the redox mode at low potentials; (2) dihydropterins are detected by fluorescence following post-column electrochemical oxidation; and (3) the oxidised pterins, which pass through the electrodes unchanged, are measured by their natural fluorescence (Fig. 5).

This direct method of pterin measurement, which requires minimal or no sample clean-up, is especially useful for the analysis of CSF where pterin concentrations are low, as differential oxidation and/or sample pre-purification leads to large errors in the estimation of the reduced pterins.

2.3.2. Other chromatographic and electrophoretic techniques

Methods other than HPLC have been used for pterin analysis. These include thin-layer chromatography (TLC) [14,78,88,105], low-voltage [106] or high-voltage [14,38,71] one-dimensional [14,106] or two-dimensional [38,71] electrophoresis, gas chromatography–mass spectrometry (GC–MS) [72] and a mixture of column and paper chromatography [11].

These methods are largely of historical interest and are rarely used in the clinical laboratory as they have inherent problems of lack of sensitivity and difficulty in quantitation. However, in the research laboratory the techniques still provide a useful tool, especially in situations where there is the possibility of the presence of an unusual pterin which might not be detected using routine HPLC methodology.

2.3.2.1. Thin-layer chromatography. TLC has been used to measure dihydroxanthopterin in urine from patients with classical phenylketonuria due to phenylalanine hydroxylase deficiency and DHPR deficiency [14,88,107] where the concentration of this compound is elevated. Urine was added without prior purification to cellulose plates and dihydroxanthopterin quantified as xanthopterin by its natural fluorescence, following separation and air oxidation at 100°C. Dihydroxanthopterin can arise from the oxidation of tetrahydro- and dihydropterins following the loss of the alkyl side-chain [65,108,109] but in phenylalanine hydroxylase deficiency and DHPR its origins are unclear. The elevation of dihydroxanthopterin is no longer used to diagnose or monitor phenylalanine hydroxylase deficiency or DHPR deficiency and no studies have been conducted to determine exactly how its concentrations are affected in these diseases. With the advent of a method for direct detection of dihydroxanthopterin by HPLC [48,60,67] this may now be possible.

A similar TLC method to that used to identify dihydroxanthopterin has been used for the separation of urinary pterins prior to quantitation by bioassay using *Crithidia fasciculata* [105].

2.3.2.2. Electrophoresis. Early studies of pterins in urine from patients with inborn errors of pterin metabolism were conducted using high-voltage electrophoresis [14,38,71]. The method requires application of urine to the paper under a stream of nitrogen. To protect reduced pterins from oxidation the paper can be soaked in N-acetylcysteine. Depending on the pterin of interest either one- or two-dimensional electrophoresis can be performed. The first dimension is run in formic acid–acetic acid–water (21:75:904, v/v/v) [1,8] or (26:120:850, v/v/v) [38,71] at pH 1.9 for 25 min at between 4000 and 5200 V. This single dimension is able to separate dihydroxanthopterin [14,38] but is unable to separate neopterin and biopterin. A second-dimension run in 1-butanol–acetic acid–water (20:3:7, v/v/v) for 3 h achieves this separation [38,71]. Papers are dried at 100°C and pterins visualised as yellow or blue fluorescent spots under UV light at 366 nm.

2.3.2.3. Gas chromatography–mass spectrometry. GC–MS has been used for the analysis of trimethylsilyated pteridines [72,110–112]. Oxidised neopterin and biopterin in human urine have been analysed by GC–MS [72] following a two-step ion-exchange sample purification procedure similar to that previously described. Other workers [89] have, however, found that trimethylsilyl derivatives of neopterin and biopterin exhibit adsorption effects on glass capillary columns and in the GC–MS interface. Also non-linear calibration curves were found which had different slopes when comparing those from the pure reference solutions and those from urines.

The sample clean-up, the complicated methodology and the problems with quantitation prevent this technique being for routine used pterin analysis. An extensive study of synthetic reference trimethylsilylated pteridines has now been conducted using glass capillary GC–MS [112] which may provide information if the structure of a pterin has to be elucidated.

2.3.2.4. Low-pressure column and paper chromatography. The first detailed quantitative separation of pterins in human urine was performed using a mixture

of traditional low-pressure column and paper chromatography [11]. Separation was performed using two weak ion exchangers (ECTEOLA-Sephadex and phospho-Sephadex) with quantitation by UV extinction coefficients or by fluorescence. Paper chromatography was used to confirm peak identity. Initially all the pterins identified were fully oxidised but a change from phospho-Sephadex to Sephadex allowed detection of BH2 and dihydroneopterin. The report demonstrates the ease by which reduced pterins may be oxidised.

2.4. Other methodologies

Several, clinically useful, non-chromatographic non-electrophoretic methods have been described for the analysis of pterins. Each is designed to measure a single pterin or a single group of pterins.

2.4.1. Biological assay

The insect parasite *Crithidia faciculata* has a nutritional requirement for biopterin [113] and this requirement forms the basis of a biological assay for total biopterins [114–117] in human serum, plasma, CSF and urine [93,105,118]. In the U.K. it is used to distinguish between patients with phenylalanine hydroxylase deficiency and BH4 deficiency after they have been shown to have a raised blood phenylalanine concentration on routine Guthrie analysis [93].

Although the *Crithidia* assay is suitable for the analysis of a large number of samples, there are several inherent problems with the method. Several pterins other than biopterin can serve as growth factors for *Crithidia* [105] and hence misleading results may be obtained if any of these are present. Because of this non-specificity, results are often expressed as "biopterin equivalents" or "*Crithidia*-active species". Samples also need to be autoclaved and then incubated for several days, during which time oxidation and loss of biopterins can occur [66]. *Crithidia* assay consistently gives lower values than those found using HPLC, presumably due to loss of reduced biopterins during the assay [119]. This loss can be completely prevented if samples are oxidised prior to autoclaving and incubation [66].

Although of value in a centralised screening laboratory, the *Crithidia* assay is of little use in a more general laboratory as it is non-specific and cannot be used to measure pterins other than biopterin.

2.4.2. Phenylalanine hydroxylase cofactor activity

BH4 concentrations in tissues and CSF have been estimated by measuring its cofactor activity for purified phenylalanine hydroxylase (EC 1.14.16.1). The assay is quantitated by monitoring either tyrosine production [3] or release of tritiated water from tritiated phenylalanine [52,120]. Problems exist with the method as q-BH2 and tetrahydrofolate also have activity within the assay system.

2.4.3. Radioimmunoassay

Specific antisera to D-*erythro*-neopterin [68,121,122], L-*erythro*-biopterin [53,91] and L-*erythro*-tetrahydrobiopterin [123] have been obtained from rab-

bits and sheep, and radioimmunoassays (RIA) using these have been developed for the measurement of biopterin and neopterin in tissues, serum and urine. The antisera to BH4 has so far only been used for immunohistochemical investigation [123].

RIA has many advantages if only neopterin or biopterin are to be measured as many samples can be assayed simultaneously. Only small volumes of sample are required, and in many cases, pretreatment of samples, other than by oxidation, is unnecessary. RIA of neopterin in serum and urine is used by many groups for monitoring diseases in which there is stimulation of the immune system. A commercial kit for RIA determination of neopterin is now available [124].

3. PTERIN MEASUREMENT IN THE DIAGNOSIS AND MONITORING OF DISEASE

3.1. Diagnosis of atypical forms of phenylketonuria

It is important to distinguish between the hyperphenylalaninaemia caused by phenylalanine hydroxylase deficiency and that caused by defects of BH4 metabolism as the clinical management of the two types of disease is very different. Phenylalanine hydroxylase deficiency is treated by the use of a low-phenylalanine diet whereas BH4 deficiency, because of the severely reduced serotonin and dopamine synthesis, requires additional therapy to overcome the neurotransmitter deficit. This is achieved by administration of the neurotransmitter precursors, 5-hydroxytryptophan and L-DOPA together with carbidopa to prevent their peripheral decarboxylation [125,126].

Two procedures have been used extensively to screen for inborn errors of BH4 metabolism. The first uses the *Crithidia fasciculata* bioassay and measures *Crithidia*-active materials in either plasma [127,128] or whole blood collected on Guthrie cards [93]. The second measures biopterins and neopterins by fluorimetry in urine after chemical oxidation and HPLC separation [70,76,85,86,89,129].

Whether using the *Crithidia* assay or HPLC, differentiation betwen phenylalanine hydroxylase deficiency and BH4 deficiency, and between the various forms of BH4 deficiency, relies on the change in pterin pattern that occurs in the different conditions. In GTP cyclohydrolase deficiency, where there is an inability to convert GTP to dihydroneopterin triphosphate [36,39], very little BH4 synthesis occurs and both neopterin and biopterin concentrations are low. The neopterin/biopterin ratio in urine remains normal [36,39]. The *Crithidia* assay also gives very low values for biopterin [36]. Absolute confirmation of GTP cyclohydrolase deficiency can now be made by enzyme assay [29].

In defects of the BH4 synthesis pathway after GTP cyclohydrolase, there appears to be a normal production of dihydroneopterin triphosphate but a failure to convert this to BH4. This results in an accumulation of neopterin and a deficiency of biopterin and consequently a large increase in the neopterin/biopterin ratio in the urine [70,76,86,89]. When an increased neopterin/biopterin ratio is found in urine it is important that the biopterin concentration is decreased, as a high neopterin concentration in the presence of a normal biopterin concentration can be found if the patient has any condition which might lead to a change in the

status of the immune systsem [49]. Concentrations of *Crithidia*-active materials in plasma in these defects are also low [127,130]. A direct enzyme assay of pyruvoyl tetrahydropterin synthetase and sepiapterin reductase are available [34,131].

In DHPR deficiency the BH4 synthetic pathway is unaffected; however, the biopterin concentrations in urine, as measured by HPLC [70,76,85,89,129], and in plasma [126,127] and blood spots [93], as measured by *Crithidia*, are raised. Neopterin concentrations are unchanged [70,85,86]. Biopterin accumulates as DHPR is not present to recycle q-BH2 back to BH4 following hydroxylation of the aromatic amino acids. The q-BH2 is unstable and rapidly undergoes tautomerisation to form 7,8-BH2 [132]. This pterin has been shown to accumulate in urine and CSF from DHPR-deficient patients [15,48]. Confirmation of DHPR deficiency may be made by measurement of enzyme activity in red cells [133] or dried blood spots [134].

A complication arises when trying to distinguish between DHPR deficiency and phenylalanine hydroxylase deficiency as hyperphenylalaninaemia, in a manner which is as yet not fully understood, itself leads to a rise in plasma and urinary biopterin [135,136]. A study in the rat suggests that the increase is due to increased synthesis from GTP [137], but it is also possible that the plasma accumulation is due to outflow from the liver [135]. Differentiation between the two can be made using the *Crithidia* assay by monitoring plasma biopterin as the phenylalanine concentration in the plasma is reduced by dietary means. A persistantly high biopterin in the face of normal plasma phenylalanine demonstrates that the patient has DHPR deficiency. Differential iodine oxidation of biopterins in urine also allows the two diseases to be separated as BH4 concentrations are negligible and BH2 concentrations high in DHPR deficiency whereas BH4 concentrations are elevated in phenylalanine hydroxylase deficiency [70,85,90].

A summary of the neopterin and biopterin changes expected in patients with the various forms of BH4 deficiency and phenylalanine hydroxylase deficiency is demonstrated in Fig. 7.

3.2. Neopterin measurement in disease

Increased concentrations of neopterin and dihydroneopterin are found in serum, CSF and urine taken from patients with a wide variety of malignant and non-malignant conditions in which there is a change in the state of the cell-mediated immune system [47,48,138]. Because neopterin concentrations are found to be raised in many conditions, the use of neopterin measurement as a "specific" diagnostic tool is of limited value. Neopterin concentrations may be significantly increased in a particular disease state compared to controls but there is generally a large scatter and an overlap between the two groups [138]. Serial measurement of neopterin concentrations in a particular patient may, however, be useful in monitoring the course of a condition.

The applications where it has been suggested that neopterin measurement may be of use include: follow up of allograft patients [139], prognostic information of cervical cancer [140], differentiation between inflammatory and degenerative

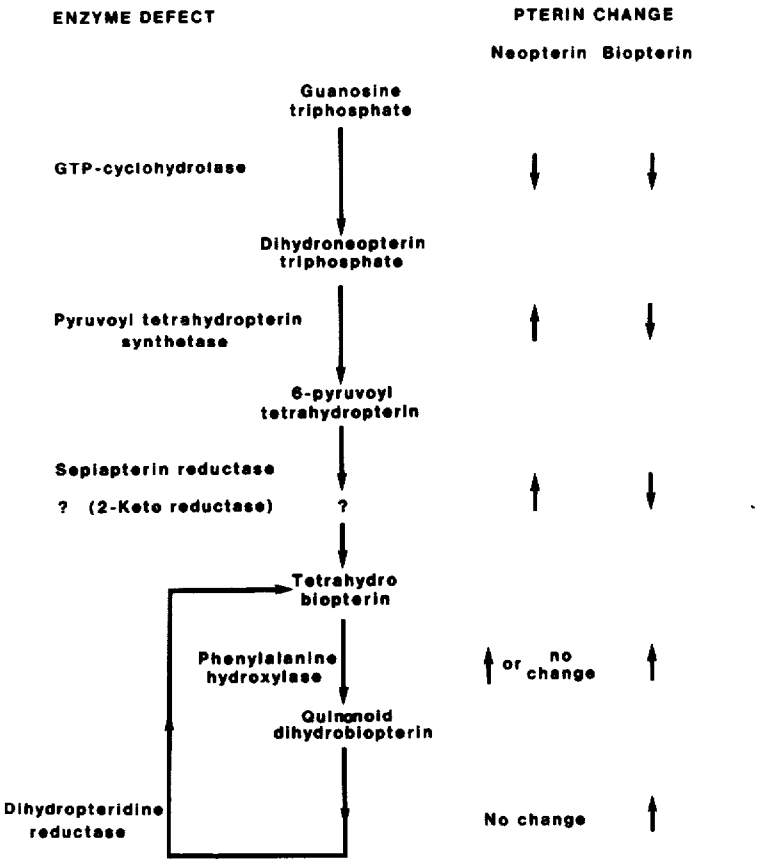


Fig. 7. Pterin changes associated with inborn errors of tetrahydrobiopterin metabolism and of phenylalanine hydroxylase.

rheumatoid disease [141], predicting activity of disease in ulcerative colitis and Crohns disease [142,143], monitoring the course of coeliac disease following commencement of a gluten-free diet [144] and as a marker for disease activity in multiple sclerosis [145] (for review see ref. 47). Also in many infectious diseases, whether caused by viruses [146], bacteria [147] or parasites [148], urinary neopterin concentrations are raised and sequential neopterin measurement can be used to monitor a course of treatment.

Although there are a mass of publications detailing the changes of neopterin which occur in a wide variety of diseases, there is in most cases no *precise* information explaining how these changes may be used to guide clinical decisions or whether the measurement of neopterin can add significantly to the information which may be obtained by other clinical or biochemical tests.

One area where neopterin measurement may provide useful data before any other tests is in the monitoring of the immune system status of transplant patients. Here early recognition and appropriate treatment of graft versus host disease or infections is essential for a successful outcome.

3.2.1. Neopterin in the follow up of transplantation

3.2.1.1. Bone marrow transplantation. After bone marrow infusion, engraftment of the transfused donor cells is followed by the presence of new peripheral blood cells two to four weeks after transplantation. A small rise in neopterin in plasma may precede the clinical detection of new cells by three to five days [149]. After the initial "take" the course of the following events may be either uncomplicated, in which case the neopterin values return to the pre-transplant values, or complicated by the onset of chronic or acute graft versus host disease, viral infection or other forms of infection which occur because of the suppressed immune state of the patient. It is important to distinguish between the various complications at an early stage as the clinical management is different for each.

The clinical manifestations of the above problems are all preceded, by up to two days, by a rise in both urinary [150] and serum neopterin [151,152]. Although a neopterin rise can foretell the onset of complications, as yet it cannot predict which of the possible problems is going to develop. With more information this may, however, be possible. There are preliminary data showing that neopterin excretion can be used to differentiate between cyclomegalovirus-induced interstitial pneumonia and idiopathic interstitial pneumonia [150] and that the kinetics of neopterin accumulation may discriminate between graft versus host disease and infectious complications [153].

3.2.1.2. Renal transplantation. The same problems exist when neopterin measurement is used to evaluate the clinical course of the engraftment following kidney transplantation. However, the interpretation of the changes of neopterin concentrations is further complicated as glomerular filtration influences urinary and serum levels of neopterin [154]. Under conditions where the kidney is not functioning well and serum creatinine concentrations are high, urinary neopterin cannot be used to predict rejection episodes [155]. Despite the problems, neopterin measurement has proved useful. An early study demonstrated that over 95% of rejection crises could be predicted by an increase in urinary neopterin and that the rise preceded the clinical signs by an average of two days [140]. Other studies have found a similar rise in both serum [156] and urine [155] prior to rejection episodes but were unable to use the rise to distinguish between actual rejection and possible infection-mediated immunological changes. The very large rise in urinary neopterin concentration seen prior to cytomegalovirus infection [155] may be useful in diagnosis of this infection.

The most beneficial use of neopterin monitoring appears to be in its ability to demonstrate that the course of the transplantation is proceeding normally. Falling neopterin concentrations following transplantation suggest that there is no infection and that rejection episodes are not occurring, hence the frequency of invasive graft biopsy and steroid therapy can be diminished [156]. In addition a return of neopterin to low levels following infection or rejection crises can be used to demonstrate the effectiveness of therapy earlier than other parameters such as serum creatinine or diuresis [156].

The available data for both renal and bone marrow transplantations suggests that sequential neopterin measurements in urine and serum following transplantation are of clinical value but they must still be used in conjunction with other

clinical and biochemical tests to ensure that a correct interpretation of the results are made.

With more studies the changes in neopterin that occur following transplantation during the normal engraftment process, during graft versus host disease and during infections may be better characterised and provide more specific data useful to the clinician for the day-to-day management of the patients. Controlled collection of urine and serum samples under conditions which do not allow loss of dihydroneopterin and measurement of both the oxidised and dihydroneopterin may allow more precise interpretation of the neopterin changes.

4. SUMMARY

This review briefly describes the biochemistry of pterins, their involvement in pathological processes and the use of pterin measurement in diagnosis and monitoring of disease. Chromatographic and other methods of pterin analysis are detailed with particular emphasis being placed on the need for correct sample collection and handling.

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