Journal of Chromatography, 381 (1986) 285—294
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3218

ESTIMATION OF TETRAHYDROBIOPTERIN AND OTHER PTERINS IN CEREBROSPINAL FLUID USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL AND FLUORESCENCE DETECTION

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(First received December 13th, 1985; revised manuscript received April 3rd, 1986)

SUMMARY

We describe an isocratic, reversed-phase high-performance liquid chromatographic method for the simultaneous measurement of fully oxidised, dihydro- and tetrahydropterins in cerebrospinal fluid. Tetrahydrobiopterin is detected electrochemically using an ESA Coulochem detector in the redox mode. Dihydropterins are detected by fluorescence following post-column electrochemical oxidation, and fully oxidised pterins by their natural fluorescence. Apart from addition of antioxidants, no sample preparation is required. Comparison is made with methods requiring chemical oxidation for detection of tetrahydrobiopterin. Some results on children with neurological disease are presented.

INTRODUCTION

Tetrahydrobiopterin (BH4) is the cofactor for phenylalanine, tyrosine and tryptophan hydroxylases [1]. The latter two enzymes catalyse the rate-limiting reactions in the synthesis of catecholamines and serotonin, respectively [1]. Patients with inborn errors of BH4 metabolism were first described in the mid-1970s [2—6], and there is some evidence that alterations of BH4 metabolism occur in Parkinson's disease [7], torsion dystonia [8], Alzheimer's disease [9], hyperphenylalaninaemia due to phenylalanine hydroxylase (PH) deficiency [10- 13] and in inborn errors of folate metabolism [14, 15].

Investigation of pterin metabolism is complicated as these compounds are unstable and can exist in the fully oxidised, dihydro and tetrahydro oxidation states. Most methods of pterin analysis involve chemical conversion of reduced

0378-4347/86/$03.50

pterins into the stable, fully oxidised, fluorescent parent compounds prior to separation by high-performance liquid chromatography (HPLC) and fluorometric analysis. By differential oxidation using acid and alkaline iodide some estimation can be made of each pterin species [16], but this requires three separate chromatograms. A simpler method using manganese dioxide (MnO_2) as the oxidizing agent has been used extensively in screening for BH4 deficiency amongst children with hyperphenylalaninaemia [17]. This method measures only total amounts of biopterins and neopterins. Direct measurement of oxidised and reduced pterins based on parallel-adjacent, dual-electrode, amperometric electrochemical detection (ED) has been described [18] but it requires extensive sample preparation under anaerobic conditions to remove co-eluting compounds and to prevent loss of unstable reduced pterins.

Procedures for direct ED of BH4 have been described [19, 20]. Measurement of fully oxidised, dihydro- and tetrahydropterins, using in series dual-cell coulometric electrochemical and fluorescence detection has been used successfully for urine pterin analysis [21], and has now been refined for analysis of pterins in cerebrospinal fluid (CSF). The present paper describes this method, compares the results with those obtained using MnO_2 and iodine (I_2) oxidations, and presents some results in children with a variety of neurological disorders.

EXPERIMENTAL

Apparatus

The chromatographic system (Fig. 1) consisted of a Spectra-Physics SP8770 pump (San Jose, CA, U.S.A.), Rheodyne 7125 injector (Cotati, CA, U.S.A.), Chrompack pellicular reversed-phase (5 × 0.45 cm I.D.) guard column (London, U.K.) and an Apex 5-μm ODS (25 × 4.5 cm I.D.) reversed-phase column (Jones Chromatography, Llanbradach, U.K.). Detection was by an ESA Coulochem 5100A electrochemical detector using a Model 5011 high-sensitivity electrode (Bedford, MA, U.S.A.) with electrodes 1 and 2 set at —0.2 and —0.5 V, respectively (gain 10 × 50, 1 V output to integrator). Downstream of this was an ESA 5021 conditioning cell (+1.0 V) followed by a Perkin-Elmer LS3 LC spectrofluorimeter (Beaconsfield, U.K.). Excitation and emission wavelengths

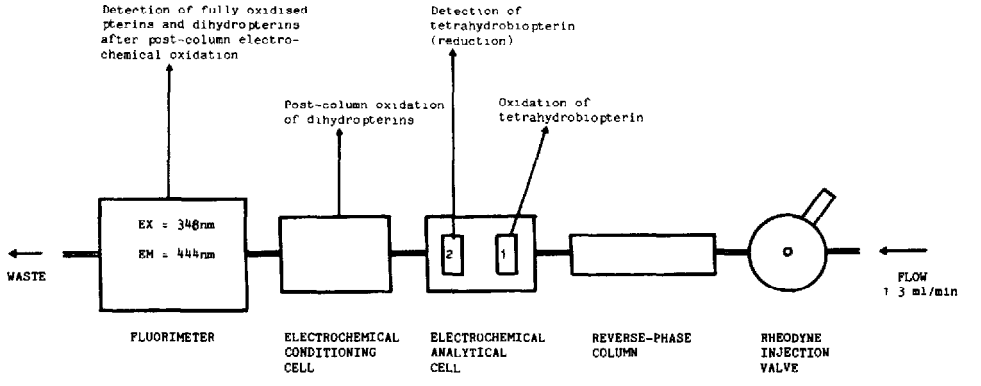


Fig. 1. Equipment configuration.

were set at 348 and 444 nm, respectively. Peak areas were determined using a Spectra-Physics SP4270 computing integrator.

Chemicals

All pterin species were obtained from Dr. B. Schircks, (Jona, Switzerland). Dithioerythritol (DTE) was purchased from Aldridge (Gillingham, U.K.). Diethylenetriaminepentaacetic acid (DETAPAC) was from Sigma (Poole, U.K.). All other chemicals were Analar grade.

Mobile phase

Sodium acetate (6.8 g), citric acid (1.05 g) and EDTA (20 mg) were added to 800 ml of distilled water. The pH was adjusted to 5.22 with sodium hydroxide or glacial acetic acid and the final volume made up to 1 l using distilled water. The mobile phase was prepared daily and filtered under reduced pressure using a 0.45-μm aqueous membrane filter (Millipore GVWP). DTE (24.7 mg/l) was added just before use. The mobile phase was degassed with helium during the chromatographic analysis. The flow-rate was 1.3 ml/min and the column temperature was maintained at 25°C.

Samples

The second 1-ml sample of lumbar CSF was collected directly into a vial containing 1 mg of DTE and 1 mg of DETAPAC, mixed and placed immediately on dry ice. Samples were stored at −70°C until analysed and were protected from light at all times; 25 μl of CSF were injected onto the column.

Oxidation of reduced pterins

Manganese dioxide oxidation was carried out as previously described [17] with modifications [15]; 25 μl of clear supernatant were injected onto the column.

Potassium iodide (KI) oxidation was carried out, with modifications [22], according to the method of Fukushima and Nixon [16]: 15 μl of 6 *M* hydrochloric acid were added to 200 μl of CSF and 0.5% I_2—1% KI in 0.1 *M* hydrochloric acid was added until excess I_2 persisted in solution. Samples were incubated at room temperature for 45 min, after which excess I_2 was reduced by addition of 1% ascorbic acid. Then 100 μl of the mixture were injected onto the column.

RESULTS AND DISCUSSION

Effects of anti-oxidants

In the presence of oxygen, BH4 rapidly autoxidises via quininoid-BH2 to 7,8-BH2 and finally to biopterin [23]. To prevent this, ascorbic acid was added to samples [21] and mobile phases deoxygenated with nitrogen or argon [18, 20]. Our preliminary experiments demonstrated that these measures were inadequate at low concentrations of BH4 (< 100 ng/ml), rapid oxidation occurring before and during the chromatographic analysis (results not shown). Combinations of ascorbic acid, mercaptoethanol, EDTA and DETAPAC also failed to prevent autoxidation of BH4 (at least 30% of BH4 was lost within 75

min at 4°C). Addition of 1 mg/ml DTE, a thiol reagent, and 1 mg/ml DETAPAC, a chelating agent to prevent oxidation catalysed by metal ions [24], protected the BH4 for at least 5 h at 4°C and for at least one month at −70°C. However, partial breakdown of BH4 still occurred on-column, forming a fluorescent product which eluted just after BH4 (not shown). As the peak symmetry of the remaining electrochemically detected BH4 was good, the breakdown product was only noticed because of the use of fluorescence detection downstream of the electrochemical cells. Addition of 0.16 m*M* DTE [19] to the mobile phase prevented this oxidation.

Measurement of BH4

BH4 was measured using the ESA Coulochem dual-electrode detector in the redox mode. BH4 was oxidised at electrode 1 (Fig. 1). Many other early-eluting compounds were also oxidised producing a large solvent front which partially obscured the BH4 peak (Fig. 2A). At electrode 2 the oxidation product of BH4 was reduced back to BH4. Non-redox compounds were not reduced, thus separating BH4 from the solvent front (Fig. 2B).

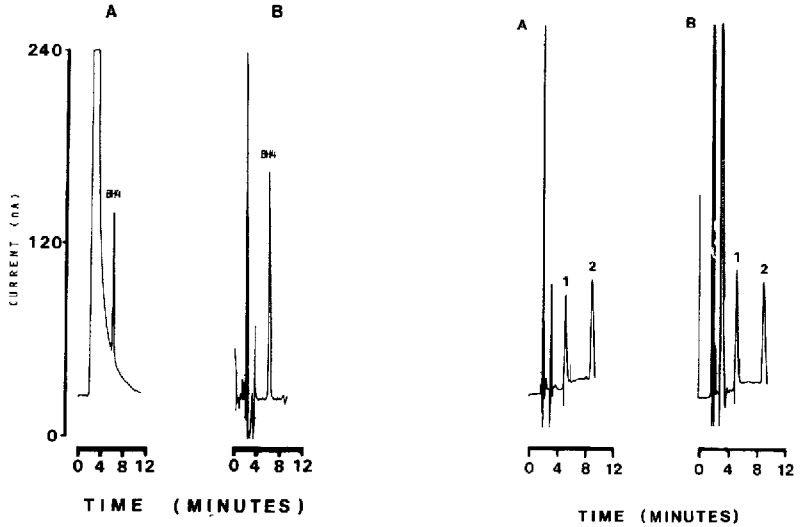


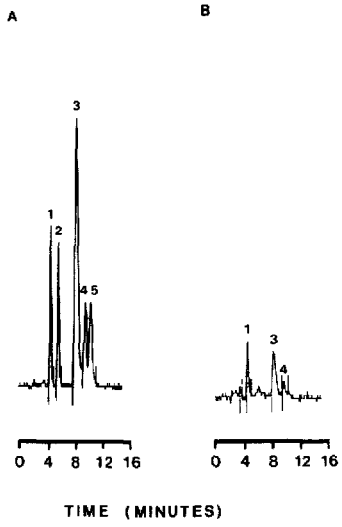
Fig. 2. Chromatograms of a tetrahydrobiopterin standard (77.5 ng/ml) using electrochemical detection. (A) Detection with an oxidising electrode only (B) Detection in the redox mode: tetrahydrobiopterin is oxidised electrochemically, then quantified on reduction back to tetrahydrobiopterin.

Fig. 3. Chromatograms of (A) a tetrahydrobiopterin standard (7.75 ng/ml) and (B) 25 μl of normal CSF Electrochemical detection. Chromatographic run at 30°C rather than 25°C. Peaks: 1 = tetrahydrobiopterin; 2 = dithioerythreitol

BH4 in CSF (Fig. 3) co-eluted with and had the hydrodynamic voltammogram of authentic BH4. It also had the same retention time as the standard material when using the mobile phase previously described for urine pterin analysis [21] (results not shown). ED of BH4 in pooled CSF which was spiked with a pterin mixture (77.5 ng/ml) gave recoveries of 95.5—98.3% (n = 5), and detection was linear up to at least 2.0 ng per injection.

Measurement of dihydro- and fully oxidised biopterins and neopterins

Fully oxidised and dihydropterins were measured by fluorescence (Fig. 4). Neopterin and biopterin are naturally fluorescent. BH2 and dihydroneopterin (NH2) are not but were electrochemically oxidised to their fluorescent parent compounds by the high oxidation potential set on the conditiong cell. Neopterin and dihydroneopterin co-elute and hence only total neopterin could be measured. However, the proportion present as dihydroneopterin was estimated by swithcing off the oxidation potential of the conditioning cell. Dihydroneopterin was no longer oxidised and the remaining peak was due to fully oxidised neopterin originally present in the sample (Fig. 5). At least 70% of total neopterins in CSF were present as dihydroneopterin. Separation of the two neopterin species could be achieved using the mobile phase described for urine pterins [21] (not shown).



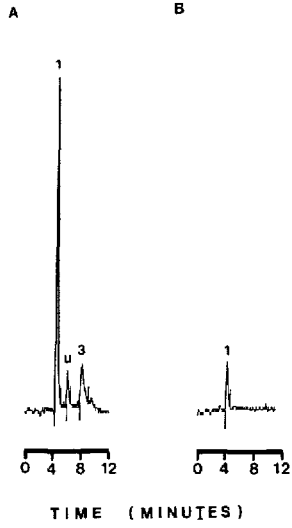


Fig. 4. Chromatograms of (A) pterin standard mixture (10 ng/ml each) and (B) 25 μl of normal CSF. Fluorescence detection. Peaks. 1 = neopterin, 2 = monapterin; 3 = dihydroxanthopterin; 4 = dihydrobiopterin; 5 = biopterin.

Fig. 5. Chromatograms of 25 μl of CSF from a patient recovering from a viral infection. (A) With electrochemical oxidation, (B) without electrochemical oxidation demonstrating the high proportion of dihydroneopterin. Fluorescence detection. Peaks as in Fig. 4; u = unidentified.

TABLE I

MINIMUM DETECTION LIMITS OF PTERIN SPECIES ANALYSED

Signal-to-noise ratio = 3.

Pterin species	Amount detected per injection (pg)
Tetrahydrobiopterin	16.9
7,8-Dihydrobiopterin	16.7
Biopterin	16 6
Neopterin	11 9

Fig. 4 shows fluorescence chromatograms of a standard and a CSF sample. The conversion of BH2 and NH2 into the fully oxidised fluorescent forms was proportional to the electrochemical oxidation potential. By varying the applied potential at the conditioning cell and monitoring fluorescence a "fluoramogram" was produced [21]. The fluoramograms for the BH2 and NH2 peaks in CSF matched those of the authentic compounds (not shown). Minimum detection limits of the pterins analysed are given in Table I.

Electrochemical conversion of dihydropterins into their parent compounds and fluorescence detection of biopterin and neopterin were linear up to at least 25 ng per injection. This range adequately encompasses the concentrations of pterins seen in CSF. Dihydroxanthopterin and monapterin are separated and can also be detected using this method (Fig. 4). Small amounts of monapterin (ca. 0.5 ng/ml) were seen in CSF from two patients with high CSF neopterins owing to viral infection (see below), otherwise it was seen only after chemical oxidation of CSF. Dihydroxanthopterin was regularly present in CSF but whether as a breakdown product of other pterins or serving some physiological function is not known.

Reliability and reproducibility

Approximately 50 ng/ml of the pterins of interest were added to a CSF pool containing 1 mg/ml DTE and DETAPAC. This was divided into 200-μl aliquots and stored at −70°C. Intra-day coefficients of variation ranged from 0.94 to 1.8% and inter-day variation (over one month) from 6.3 to 10.9% (Table II).

TABLE II

INTRA- AND INTER-DAY COEFFICIENTS OF VARIATION FOR NEOPTERIN, BIOPTERIN, DIHYDROBIOPTERIN AND TETRAHYDROBIOPTERIN IN A SPIKED CSF POOL

Species	Coefficient of variation (%)	
	Intra-day (n = 5)	Inter-day (n = 5)
Neopterin	0.94	7.20
Biopterin	1.79	10.9
7,8-Dihydrobiopterin	1.42	6.3
Tetrahydrobiopterin	1.27	8.6

Comparison with methods requiring chemical oxidation

BH4 (20 ng/ml) was added to a CSF pool containing 1 mg/ml DTE and DETAPAC, and 500-μl aliquots were frozen at −70° C. BH4 in each aliquot was assayed using ED and by the MnO_2 [17] and KI [22] oxidation methods. Both chemical oxidation procedures required two chromatograms for the estimation of all three pterin oxidation states, the first measuring biopterin and BH2 present in the sample before chemical oxidation and the second total biopterin after chemical oxidation. The concentration of BH4 was obtained by subtraction [total biopterin − (biopterin + BH2) = BH4], thus compounding the errors of three individual measurements. All methods gave

TABLE III

COMPARISON OF BH4 RESULTS OBTAINED ELECTROCHEMICALLY AND AFTER MnO_2 AND KI OXIDATION OF A POOLED CSF SAMPLE SPIKED WITH 20 ng/ml BH4

The concentration of tetrahydrobiopterin was calculated from [total biopterin — (endogenous biopterin + 7,8-BH2) = BH4].

Method	BH4 concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
Electrochemistry	19.00 ± 0.65	3.43
MnO_2 oxidation	15.08 ± 3.70	24.5
KI oxidation	22.47 ± 5.85	26.0

similar mean concentrations but electrochemical detection gave a much narrower range of values (Table III).

Pterin analysis in CSF from patients with neurological disorders

CSF was obtained from 33 children with various neurological disorders. Of these, two had phenylketonuria due to phenylalanine hydroxylase deficiency (patients 1 and 2), one phenylketonuria due to dihydropteridine reductase deficiency (patient 3), and two post-viral neurological illness (Herpes simplex encephalitis, patient 4, and probable intrauterine viral infection, patient 5). One child who died in the neonatal period (patient 6) had non-ketotic hyperglycinaemia, another had B12 responsive methylmalonic aciduria and two brothers had dibasic aminoaciduria. The remainder had neurological illness of unknown cause. Most concentrations of BH4 and total neopterin fell between 1 and 10 ng/ml (Fig. 6). BH2 was seen in over half the specimens but fully oxidised biopterin was detected only in patient 3. Eight patients (patients 1 to 8) had pterin values above the 1—10 ng/ml range.

Patients 1 and 2 (phenylalanine hydroxylase deficiency). These were both on a low phenylalanine diet, which was better controlled in patient 2 (plasma phenylalanine 300 μmol/l) than patient 1 (plasma phenylalanine 900 μmol/l). Most of the total biopterin in CSF was present as the tetrahydro form and the concentration of BH4 in both patients was elevated. BH2 values were at the upper limit of the control range. BH4 and BH2 concentrations were lower in patient 2 than patient 1, who had higher plasma phenylalanine concentrations and a markedly increased total neopterin. Fully oxidised biopterin was not detected. These data are in agreement with previous reports showing increased biopterins and neopterins in proportion to the hyperphenylalaninaemia in the urine and blood of patients with phenylalanine hydroxylase deficiency [10, 12, 25—28] and are consistent with in vitro studies showing stimulation of BH4 synthesis from guanosine triphosphate by hyperphenylalaninaemia [29].

Patient 3 [dihydropteridine reductase (DHPR) deficiency]. Patients with DHPR deficiency can synthesise BH4 de novo but DHPR is required for the salvage of quinonoid dihydrobiopterin to BH4 [1]. These patients accumulate biopterins in blood [28], urine [25] and CSF [15, 30], and urine pterin analysis has shown that they excrete mainly BH2 [21]. Previously, using the MnO_2 oxidation procedure, the increased total biopterins in CSF were also

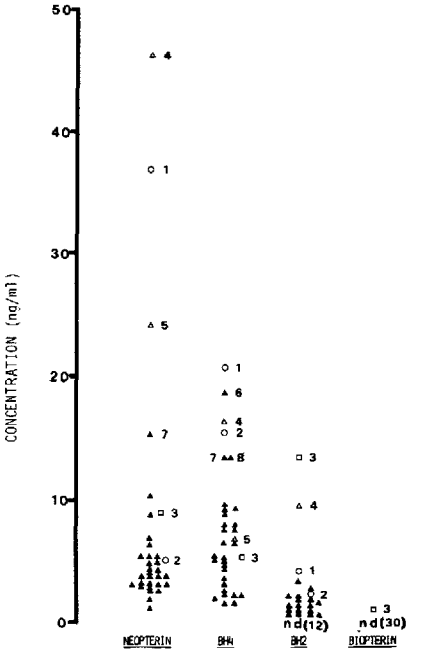


Fig. 6. CSF pterin results from patients with neurological illness. (○) Phenylalanine hydroxylase deficiency; (□) dihydropteridine reductase deficiency; (△) post-viral neurological illness; (▲) other patients with neurological illness. Numbers refer to patients detailed in text; nd = not detected.

found to be mainly BH2 with no BH4 detected [15]. The patient investigated here showed increased BH2 and biopterin. However, BH4 (5.4 ng/ml) (Fig. 6) was also present at normal concentrations. This finding is consistent with the suggestion that salvage of BH4 continues in the absence of DHPR activity [15, 30]. It has been proposed [15, 30] that the folate enzymes, 5,10-methylenetetrahydrofolate reductase and dihydrofolate reductase, convert quinonoid dihydrobiopterin and 7,8-dihydrobiopterin, respectively, into BH4 [31–33]. However, any salvage of BH4 in the central nervous system (CNS) cannot be occurring in the correct physiological compartment since the patient also exhibited typical clinical symptoms with profound deficiency of neurotransmitter amines [15, 30].

Patients 4 and 5 (Herpes simplex encephalitis/intrauterine viral infection). Concentrations of neopterins are known to increase in blood and urine in association with viral infection owing to stimulation of macrophages by lymphokines released from activated T-cells [34, 35]. The present results show that an increase in total neopterin, due predominantly to NH2 (Fig. 5), also occurs in the CNS in response to viral disease, perhaps due to invasion by macrophages. Patient 4 had raised BH4 in association with the raised neopterin, suggesting increased synthesis of BH4, not just of neopterins.

Patients 6, 7 and 8. These three patients had high BH4 concentrations, which at present cannot be explained. Patient 6 had hyperglycinaemia and died in the neonatal period. Patient 7 who had liver disease and CNS disease of

unknown cause and who also died early in infancy, had a raised neopterin as well as BH4. Patient 8 had a neurodegenerative disorder leading to hypotonia and uncontrolable fits of unknown cause.

CONCLUSION

Direct analysis of pterin species demonstrates that BH4 and NH2 are the main biopterin and neopterin species in CSF and helps to clarify the changes that occur in different forms of hyperphenylalaninaemia and in viral illness.

ACKNOWLEDGEMENTS

We are grateful for financial support of the Medical Research Council and The National Fund for Research into Crippling Diseases. K.H. is in receipt of a Research Fellowship from the Robert McAlpine Foundation. We would like to thank Dr. J. Wilson, Dr. E. Brett and Dr. J.V. Leonard for allowing us to investigate their patients.

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