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Note

Estimation of tetrahydrobiopterin and other pterins in plasma by isocratic liquid chromatography with electrochemical and fluorimetric detection

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Tetrahydrobiopterin (BH_4) is the essential cofactor for the hydroxylase enzymes of phenylalanine, tryptophan and tyrosine [1]. These enzymes are involved in the synthetic pathways of various neurotransmitters, e.g. dopamine and 5-hydroxytryptamine from tyrosine and tryptophan. The metabolism of the pterin species, including BH_4, is of interest in fields of research such as Parkinson's disease [2], Alzheimer's disease (AD) [3] and phenylketonuria (PKU) [4].

Pterins exist in several oxidation states: fully oxidised biopterin and neopterin, partly reduced dihydrobiopterin (BH_2) and fully reduced BH_4 [5]. It has been possible for some time to determine the total pterin content of biological samples. This involves converting the various constituent pterins into their fully oxidised form by chemical oxidation, using either acid/iodine [6] or manganese dioxide [7], prior to separation and quantification by liquid chromatography (LC) with fluorimetric detection.

Direct determination of the dihydro and tetrahydro forms is possible using LC with electrochemical detection (LC–ED) and methods have been published which are applicable to urine and cerebrospinal fluid (CSF) [8,9]. However, problems arise when dealing with blood as BH_4 is highly labile and levels are much lower than in urine. Previous methods have also experienced difficulties with sample preparation [10].

We describe here an isocratic LC–ED method, also with fluorimetric detection, for the determination of free BH_4 as well as biopterin, neopterin and BH_2 in plasma. This is combined with a sample preparation technique which offers the advantages of being both rapid and simple.

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EXPERIMENTAL

Chromatography

Apparatus. The chromatographic system consisted of a ConstaMetric I pump (LDC/Milton Roy, Stone, U.K.) with pressure pulse dampener (Severn Analytical, Gloucester, U.K.), WISP 710B autosampler (Waters, Milford, MA, U.S.A.), S3ODS2 (125 mm × 4.6 mm I.D.) reversed-plasma column (Hichrom, Reading, U.K.), preceded by a 5-μm in-line filter, ESA Coulochem electrochemical detector with Model 5021 conditioning cell and Model 5011 analytical cell (Severn Analytical), Model 3000 fluorescence spectrophotometer (Perkin-Elmer, Beaconsfield, U.K.).

Chemicals. Pterin standards (BH_4, biopterin, neopterin, 7,8-dihydrobiopterin and 6-hydroxymethylpterin) were purchased from Dr. B. Schircks Labs. (Jona, Switzerland). Ethylenediaminetetraacetic acid (EDTA) (Analar grade), potassium dihydrogen orthophosphate (Aristar grade), orthophosphoric acid (Aristar grade) and methanol (Hipersolv) were all purchased from BDH (Poole, U.K.). Dithioerythritol (DTE) was purchased from Aldrich (Gillingham, U.K.) and octane sulphonic acid from Fisons (Loughborough, U.K.).

Mobile phase. Mobile phase was prepared daily by dissolving 10 ml of 0.1 *M* octane sulphonic acid, 20 ml of methanol, 6.8 g of potassium dihydrogen orthophosphate and 10 mg of EDTA in 800 ml of doubly distilled water. The pH was adjusted to 2.8 by the addition of orthophosphoric acid and the final volume made up to 11 with doubly distilled water. DTE (50 mg) was added just prior to use and the mobile phase filtered (0.22 μm) under reduced pressure. Chromatography was performed at a flow-rate of 1.3 ml/min and the mobile phase run to waste.

Sample preparation

Whole blood was collected into microfuge vials containing sufficient DTE and EDTA to give a final concentration of approximately 1 mg of each per ml of blood, spun for 3 min in a benchtop microfuge and the plasma transferred to an MPS-1 micropartition system fitted with M_r 100 000 cut-off membranes (Amicon, Stonehouse, U.K.). Following addition of 6-hydroxymethylpterin as an internal standard to correct for sample recovery the plasma was spun across the micropartition membrane at 2000 *g* for 45 min using an MSE 25 centrifuge (Fisons) fitted with a fixed-angle rotor and pre-cooled to 2 °C. Prepared filtrate was stored at −70 °C until analysed.

Analysis

The arrangement of the chromatographic system is shown in Fig. 1. Between 50 and 100 μl of plasma filtrate were injected for LC analysis. Precautions were taken to ensure that samples were protected from the light and only thawed out immediately prior to analysis.

BH_4 was quantified electrochemically using the Coulochem detector in redox mode, in order to achieve the required selectivity. The compound was first oxi-

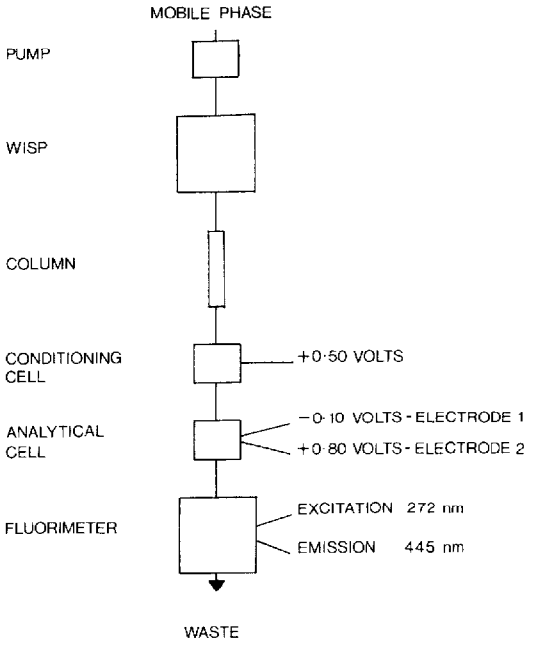

Fig. 1. Lay-out of HPLC system.

dised on the conditioning cell electrode at +0.50 V prior to being quantified by the subsequent reduction at −0.10 V on electrode 1 of the analytical cell.

Fully oxidised pterins (biopterin and neopterin) are naturally fluorescent and BH_2 can be detected fluorimetrically (excitation 272 nm, emission 445 nm) following oxidation on electrode 2 of the analytical cell. The xenon source lamp used by the Perkin-Elmer fluorescence spectrophotometer has a high intensity, providing a continuum of energy over the required excitation spectrum. The excitation wavelength given here may therefore be lower than those published for other instruments.

The fluorimetric detection of BH_2 is dependent on the compound being oxidised at a relatively high potential on electrode 2 of the analytical cell. In order to achieve full oxidation the electrode potential is adjusted to give the maximum possible peak height.

RESULTS

The peak detected in samples by ED had the same retention time as authentic BH_4 (Fig. 2A and B) and further exhibited the same hydrodynamic voltammogram as the genuine compound. Other pterins (neopterin, biopterin and BH_2) determined by fluorimetric detection had the same retention times as the authentic compounds. All detectable peaks eluted within approximately 35 min.

Recovery of BH_4 from spiked plasma had a mean of 43.9% ($n=6$) with an intra-batch coefficient of variation of 7.8%. Detection was linear to at least 30 ng per injection with a minimum detectable quantity of 0.1 ng per injection.

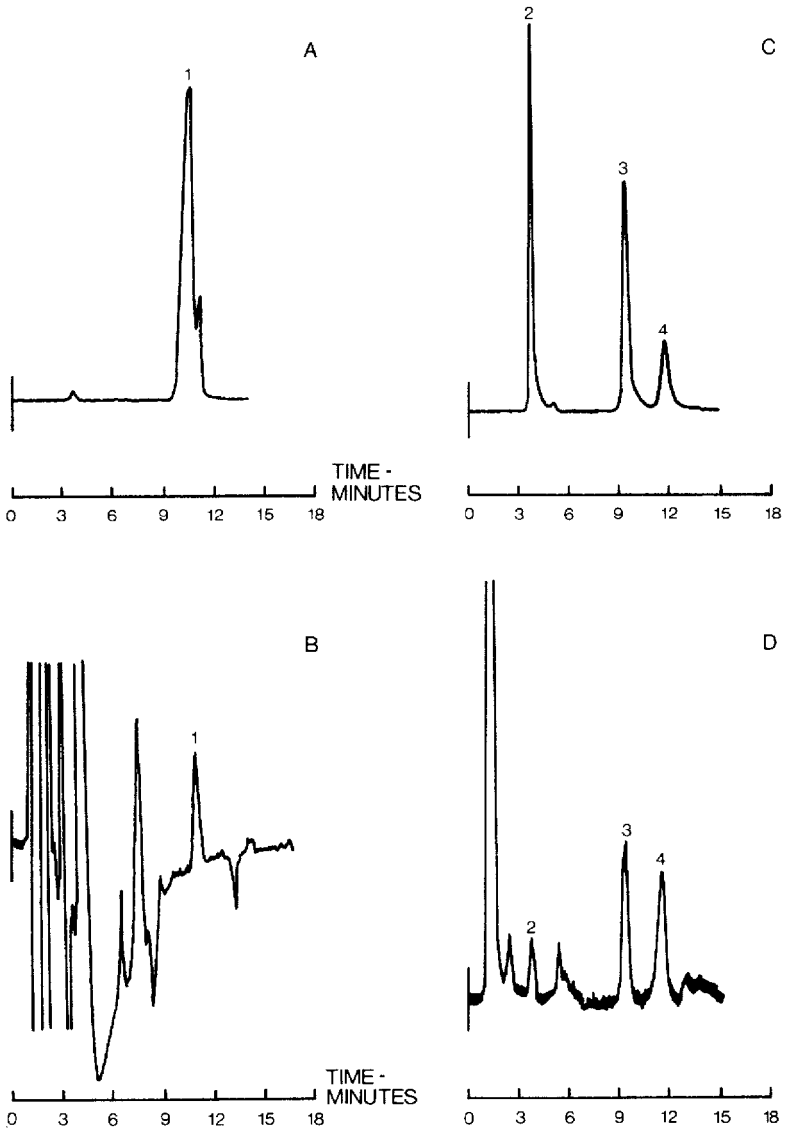

Fig. 2. Example chromatograms. (A) Electrochemical trace from 0.70 ng of authentic BH_4; (B) electrochemical trace from 75 μl of prepared plasma; (C) fluorescent trace from 1.00 ng each of neopterin, biopterin and dihydrobiopterin; (D) fluorescent trace from 75 μl of prepared plasma. Peaks: 1 = BH_4; 2 = neopterin; 3 = biopterin; 4 = BH_2.

Sample recovery was corrected by the addition of a known quantity of 6-hydroxymethylpterin as an internal standard prior to sample preparation. Recovery ranged from 61 to 65% ($n=6$) with an intra-batch coefficient of variation of 2.2% and an inter-batch coefficient of variation of 3.9% ($n=6$). Intra-batch coefficients of variation for the pterins of interest are shown in Table I.

Plasma pterin concentrations were estimated in a group of twenty healthy volunteers aged between 21 and 42 years. Results (mean $\pm$ S.D.) were:

TABLE I

INTRA-BATCH COEFFICIENTS OF VARIATION

Pterin	Coefficient of variation (%)	n
Biopterin	7.9	9
Neopterin	11.9	6
BH_2	11.2	9
BH_4	10.6	9

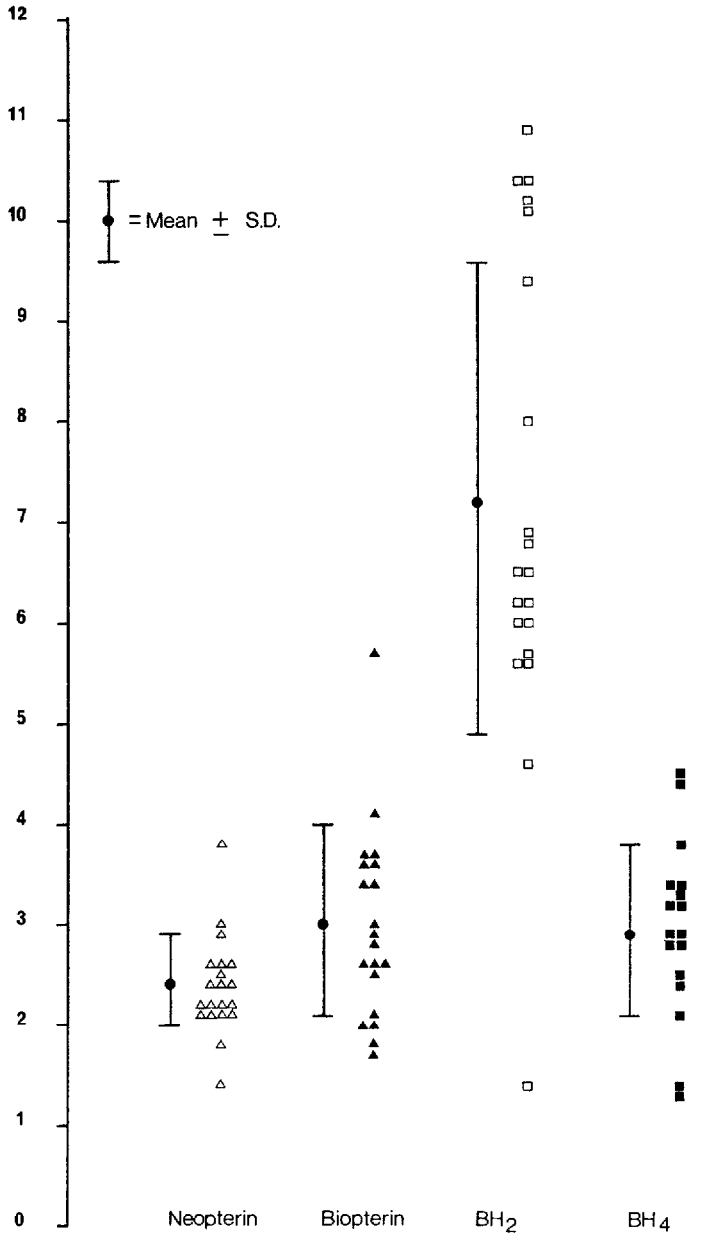

Fig. 3. Plasma pterin concentrations.

$BH_4 = 2.99 \pm 0.85$ ng/ml ($n = 17$); $BH_2 = 7.20 \pm 2.35$ ng/ml, biopterin = 3.03 ± 0.92 ng/ml; neopterin = 2.43 ± 0.48 ng/ml. The results are shown in Fig. 3.

DISCUSSION

When using the Coulochem detector in redox mode the compound is first oxidised and then quantified by the subsequent reduction. Normally these processes take place on electrodes 1 and 2 of the analytical cell, respectively. In quantifying BH_4, electrode 1 was found to give superior sensitivity to electrode 2 so the cells were arranged to oxidise the compound on the conditioning cell (Fig. 1).

It should be stressed that the electrode potentials shown were found to be suitable for our detector, but settings should be optimised for individual electrodes by the generation of hydrodynamic voltammograms. Optimum potentials may also shift and so it is advisable to regularly recheck the voltammogram.

BH_4 is a highly labile compound and will rapidly auto-oxidise to 7,8-dihydrobiopterin, quinonoid dihydrobiopterin and finally biopterin [11]. It is, therefore, necessary to use an antioxidant to preserve the specimen. DTE was found to be a suitable compound for collection and protection of samples, as reported in previous studies [8], when used in conjunction with a chelating agent such as EDTA [9]. DTE is a thiol agent, protecting –SH groups in the sample. Chelating agents prevent oxidation caused by metal ions [9], EDTA also acting as an anti-coagulant when collecting whole blood. Studies using authentic solutions of BH_4 showed the compound to be fully protected by the DTE–EDTA combination for 2.5 h with the loss of only 15% after 4 h when refrigerated at 4 °C and protected from light. This is compared with the loss of 38% of the compound after 4 h when protected by DTE alone (Fig. 4). No problems of sample degradation were experienced when frozen at −70 °C in DTE–EDTA.

Ascorbic acid was also evaluated as an antioxidant but was found to give instability in the electrochemical detector. This problem was not experienced with the use of DTE.

The use of a micropartition system provides several advantages over other methods of sample preparation. (i) It is very convenient to use, the filtrate being sufficiently deproteinised for direct LC analysis (99.9% of the plasma proteins are retained by the membrane). (ii) Other methods of sample preparation involve chemical precipitation of proteins using, for example, trichloroacetic acid (TCA). This dilutes the sample and may necessitate a concentration step which is time-consuming when handling a compound as labile as BH_4. We have also experienced stability problems with the baseline of the electrochemical detector when attempting TCA-based techniques.

Our method of sample preparation avoids these problems while providing good precision and recovery. However, it should be pointed out that protein binding of pterins occurs in the plasma, mainly by unspecific adsorption [12] and so the estimations of the pterins reported here refer to the free concentrations. 6-Hydroxymethylpterin proved to be satisfactory as an internal standard to correct for recovery. It does not normally occur in the plasma [13] but has a similar

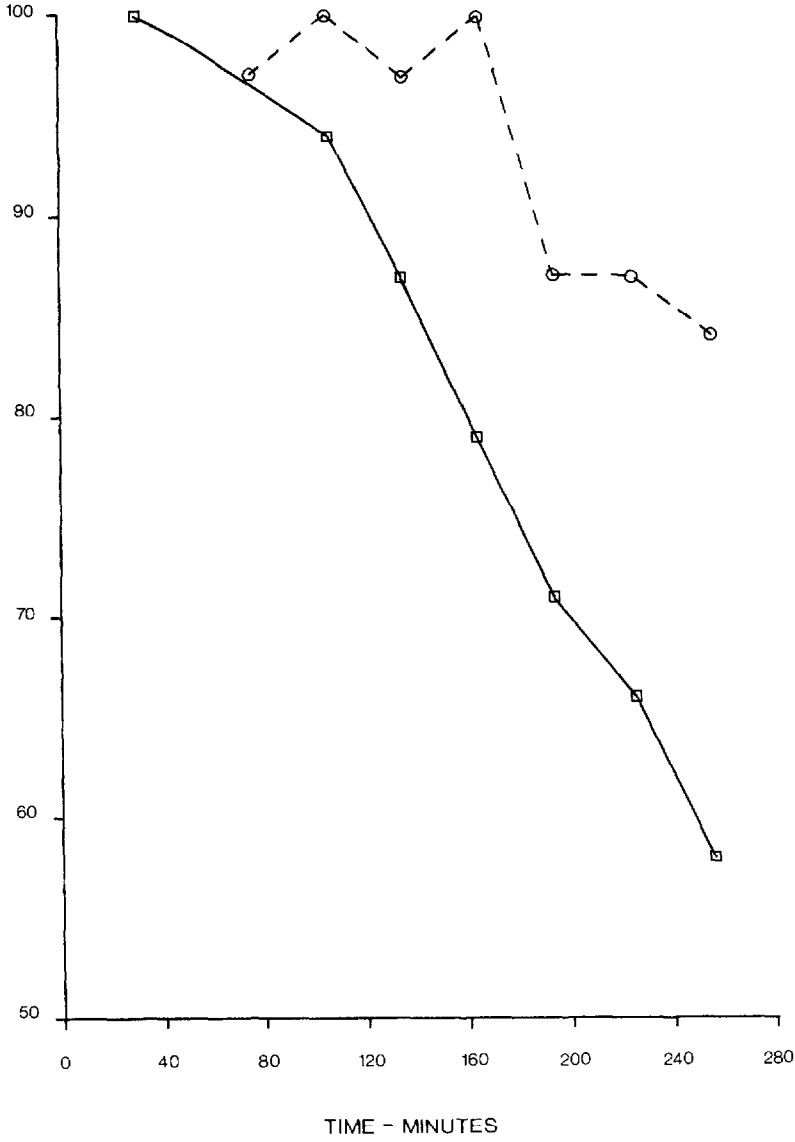

Fig. 4. Degradation of authentic BH_4 at 4°C when protected from light. (□) Sample protected by 1 mg/ml DTE; (○) sample protected by 1 mg/ml DTE and 1 mg/ml EDTA.

chromatographic behaviour to the other pterins while not interfering with their detection.

The results obtained compare with those of Fukushima and Nixon [6] who reported plasma biopterin and neopterin concentrations of 2.6, 2.3, 3.4 and 6.1, 3.3, 5.2 ng/ml, respectively, for each of three subjects. Howells et al. [9], working with CSF, found concentrations of BH_4 and total neopterin to be between 1 and 10 ng/ml.

The chromatographic procedure described is also applicable to other biological

fluids, i.e. urine and CSF. Samples are collected into DTE–EDTA mixture and frozen at −70 °C until analysed. Sample preparation is minimal with these fluids, precautionary filtration (0.22 μm) being all that is required prior to chromatography.

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