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Note

Determination of biopterin and other pterins in tissues and body fluids by high-performance liquid chromatography

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Tetrahydrobiopterin serves as a cofactor in the hydroxylation of phenylalanine, tyrosine and tryptophan [1] as well as in the cleavage of glycerol ethers [2]. Further investigation of alterations of pterin metabolism associated with phenylketonuria [3], Parkinsonism and other neurological diseases [4, 5], and neoplastic diseases [6, 7] as well as investigations related to the biosynthesis and metabolism of the biopterin cofactor necessitate a method that is specific, rapid and reproducible for analyzing these compounds in tissues and body fluids.

Biopterin and/or its metabolites and related compounds have been determined by bioassay using *Crithidia fasciculata* [8], enzymatic assay using phenylalanine hydroxylase [9], conventional column and thin-layer chromatography [10, 11], radioimmunoassay [4], and high-performance liquid chromatography (HPLC) [12, 13]. However, limitations of these methods include a lack of specificity, the need for large amounts of material, long retention times, the inability to detect all precursors and metabolites of biopterin, or changes in the elution profile due to salt effects. The aim of this investigation was to develop an HPLC method for the separation of the pterins present in mammalian tissues and fluids that is rapid, specific and insensitive to salts present in the samples to be analyzed.

MATERIALS AND METHODS

Biopterin, neopterin, sepiapterin and 6-hydroxymethylpterin were purchased from Dr. B. Schircks (Wettswil, Switzerland); pterin, xanthopterin, isoxanthopterin and pterin-6-carboxylic acid were from Sigma (St. Louis, MO,

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U.S.A.). Acetonitrile and tetrahydrofuran were HPLC grade. All other chemicals were reagent grade.

Neuroblastoma N115 cells were grown in Dulbecco's Modified Eagle Medium containing 10% fetal calf serum. Sample preparation was carried out as described by Fukushima and Nixon [12]. Urine samples were chromatographed directly following iodine oxidation in acid or base after excess iodine had been neutralized with ascorbic acid. Extracts of cells in culture were carried through the entire procedure. Creatinine was determined using a creatinine kit from Sigma according to the method of Jaffé [14] as modified by Heinegard and Tinderstrom [15].

Fluorescence spectra of the pterins were determined using a Perkin-Elmer MPF-2A scanning spectrofluorometer. The HPLC system consisted of a Tracor Model 995 isochromatographic pump, a Waters Model 710B WISP autoinjector and either a Perkin-Elmer Model 650-10LS or a Schoeffel Model FS-970 fluorometer. Data were collected and analyzed with a DS-80 microcomputer (Digital Specialties). A Whatman PXS 10/25 ODS 10-μm column (25 × 0.46 cm) fitted with a pre-column packed with CO:Pell ODS (7 × 0.21 cm) was used in all separations. The solvent system consisted of 0.5% acetonitrile and 0.1% tetrahydrofuran in water which was filtered using a 0.45-μm Ultipor NX membrane filter (Rainin), and degassed before use. Flow-rate was 1.5 ml/min. Pterins were detected with the Perkin-Elmer using an excitation wavelength of 360 nm with a 5-nm slit width and an emission wavelength of 450 nm with a 10-nm slit width. When the Schoeffel fluorometer was used, a filter with an emission cutoff below 418 nm was employed.

RESULTS AND DISCUSSION

The fluorescence spectra of the oxidized pterins were determined in the solvent system used for HPLC. The excitation and emission maxima are given in Table I. Based on these results an excitation wavelength of 360 nm was chosen for detection of pterins in this system. A chromatogram illustrating the separation of a mixture of standard pterins is illustrated in Fig. 1A. The elution profile was not affected by salts or organic solvents in the sample. All samples were acidified before being chromatographed since pterin-6-carboxylic acid would elute in the void volume if the samples were at neutral or alkaline pH. The k' values as well as the limits of detection of each of the pterins are also presented in Table I. The limits of detection for all the pterins except xanthopterin ranged from 50—200 pg. However, sensitivity could be increased by the use of wider slit widths on the Perkin-Elmer or by the selection of a different excitation wavelength if increased sensitivity for a specific pterin is desired. During the useful life of the column retention times did not significantly vary and repeated injections of standard solutions of biopterin indicated a variation in peak heights of 4%.

The limits of detection of xanthopterin are considerably higher than those of the other commonly occurring pterins, due to the fact that xanthopterin elutes as a broad peak and also has an excitation maximum considerably higher than the other pterins. Under the elution conditions employed in this study, sepiapterin is markedly retained on the column. Moreover the marked

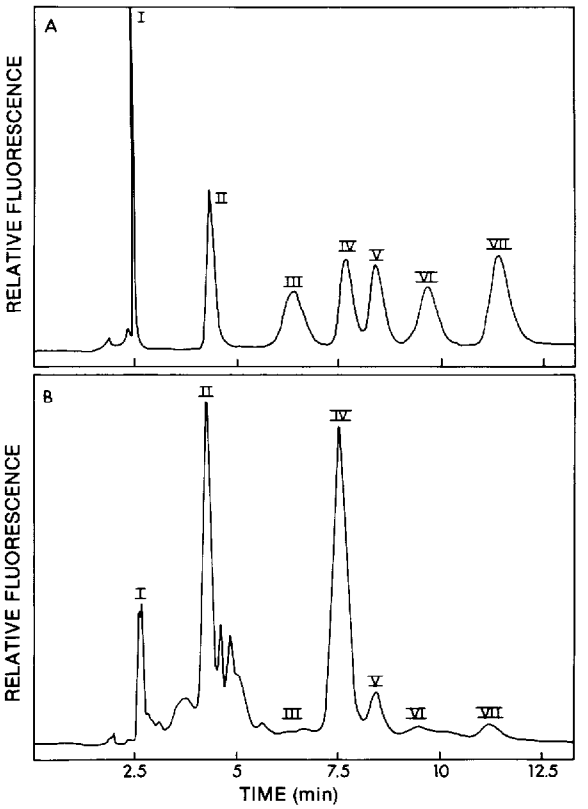

Fig. 1. HPLC separation of pterins. (A) Chromatogram of a standard mixture of pterins containing 56 ng of xanthopterin and 2.25 ng of all the other pterins. (B) Chromatogram of normal human urine that had been oxidized with iodine in acidic solutions and the excess iodine removed by addition of ascorbic acid. The equivalent of 10 μl of urine was chromatographed. Peaks: I = pterin-6-carboxylic acid; II = neopterin; III = xanthopterin; IV = biopterin; V = isoxanthopterin; VI = 6-hydroxymethylpterin; VII = pterin.

difference in excitation and emission maxima from those used in the present study renders all but large quantities of sepiapterin undetectable.

The chromatographic system described above was used for the analysis of pterin distribution and content in human urine and neuroblastoma cells in culture. A typical chromatogram of normal human urine that had been oxidized with iodine in acid solution is presented in Fig. 1B and the distribution of pterins in normal urine as well as in neuroblastoma cells is presented in Table II. The concentration of pterins in urine is high enough and interfering fluorescent material low enough so that urine can be chromatographed directly after iodine oxidation without being further treated on Dowex resins as described for tissues [12, 13]. However, the cells in culture must be carried through the entire procedure. Using the conditions described above the high concentration of salt present in urine samples does not affect the elution profile of the pterins, and all of the pterins which commonly occur in tissues and urine can be separated and quantitated.

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS OF PTERINS

Pterin	λ max (nm)		k'*	Limits of detection** (pg)
	Excitation	Emission		
Pterin carboxylic acid	365	444	0.32	45
Neopterin	365	450	1.29	190
Xanthopterin	397	465	2.38	2700
Biopterin	364	447	3.05	185
Isoxanthopterin	347	405	3.47	90
Hydroxymethylpterin	357	443	4.13	185
Pterin	355	442	5.04	200
Sepiapterin	420	520	—	—

*$k' = \frac{V_e - V_0}{V_0}$ where V_e is the elution volume and V_0 is the void volume.

**Excitation 360 nm; emission 450 nm.

TABLE II

PTERIN CONTENT OF BIOLOGICAL SAMPLES

Pterin	Urine: ng/mg creatinine	Neuroblastoma N115: ng/10^6 cells	
		Cells	Medium
Pterin carboxylic acid	3.2 ± 0.5	n.d.*	n.d.
Neopterin	539 ± 90	0.079 ± 0.01	4.14 ± 0.88
Xanthopterin	258 ± 91	n.d.	n.d.
Biopterin	1148 ± 76	8.13 ± 0.88	20.1 ± 1.9
Isoxanthopterin	1007 ± 234	n.d.	n.d.
Hydroxymethylpterin	25.4 ± 4.7	0.257 ± 0.034	29.8 ± 7.5
Pterin	126 ± 31	1.57 ± 0.17	119 ± 16.7

*n.d. = Not detectable.

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