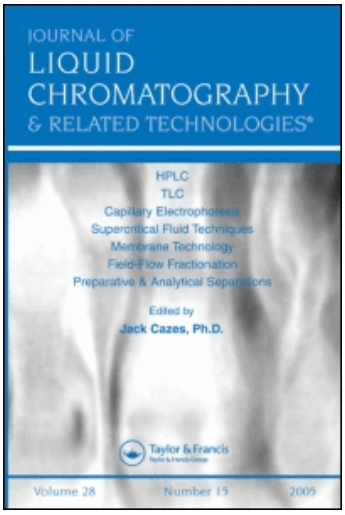

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DETERMINATION OF THE OXIDIZED AND REDUCED FORMS OF BIOPTERIN IN TISSUE SAMPLES

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ABSTRACT

Biopterin, in its reduced form, is a cofactor to the enzymes catalyzing the rate-limiting reactions in the synthesis of both the catecholamines and serotonin. It has been suggested that it may serve a role in the regulation of these neurotransmitters. Altered biopterin concentrations have also been reported in various neural disorders. In this report, we describe a method using liquid chromatography/electrochemistry to quantitate both the oxidized and reduced forms of biopterin in mouse tissue samples. This method employs a dual-electrode amperometric detector in the parallel-adjacent configuration. Subpicomole detection limits were achieved for all oxidation states.

INTRODUCTION

L-*erythro*-tetrahydrobiopterin (Figure 1) has been shown to be a cofactor in several hydroxylation reactions. These include the hydroxylation of phenylalanine (1), tryptophan (2) and tyrosine (3). Recently, there has been speculation that the concentration of tetrahydrobiopterin may serve a function in the regulation of these reactions (4). In order to elucidate the role of tetrahydrobiopterin and to study the mechanism of its function, a method is needed to quantitate it at the picomole level in various tissue samples.

Several methods of determining biopterin concentrations are available: a bioassay using *Crithidia fasciculata* (5), a radioenzymatic assay (6),

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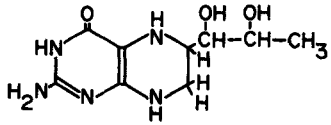
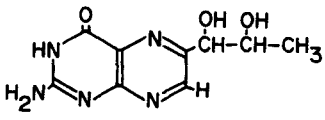
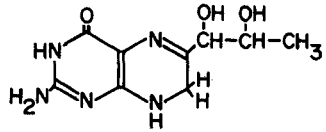


5,6,7,8-TETRAHYDROBIOPTERIN



BIOPTERIN



7,8-DIHYDROBIOPTERIN

Figure 1. Structures of the oxidized and reduced forms of biopterin.

an immunoassay (7), and liquid chromatography with fluorescence detection (8,9). None of these methods can directly detect all of the oxidation states of biopterin. We have recently reported a method employing liquid chromatography/electrochemistry (LCEC) for the determination of a variety of pterin species and oxidation states in urine (10). Bräutigam and Dreesen have also described the detection of tetrahydrobiopterin by LCEC (11). In this report, a method using LCEC with a dual-electrode detector is described for the determination of the stable oxidation states of biopterin in tissue samples.

EXPERIMENTAL

Chemicals. All chemicals were reagent grade or better. Biopterin was purchased from Calbiochem-Behring (La Jolla, CA). Octyl sodium sulfate was obtained from Eastman Kodak Co. (Rochester, NY). Tetrahydrobiopterin was a gift of Dr. A. Niederweiser (Zürich, Switzerland). Tetrahydrobiopterin and 7,8-dihydrobiopterin were prepared as described previously (10).

Apparatus. The chromatography system employed consisted of an Altex 110 pump and dual LC-4B amperometric detectors (Bioanalytical Systems, West Lafayette,

IN). Chromatography was performed on a Biophase ODS 5μ column (25 cm x 4.6 mm). The column was thermostated at 30°C by an LC-23 column heater and an LC-22 temperature controller (BAS). A 20 μL injection loop was employed.

Liquid Chromatography. An "ion-pair" reverse-phase chromatographic system was used to achieve separation. The mobile phase was 3 mM octyl sodium sulfate in a 0.1 M sodium phosphate buffer, pH 2.5, with 15% methanol. A standard separation is shown in Figure 2. The mobile phase was prepared from distilled, deionized water and filtered through a 0.22 μm filter (Millipore, Milford, MA). Oxygen was removed by continuous purging of the mobile phase with nitrogen and maintaining the mobile phase reservoir at a temperature of 40°C. A flow rate of 1.0 mL/min was used for all experiments.

Sample Preparation. Mice were sacrificed by decapitation. The organ of interest was removed, weighed wet, and homogenized in 0.1 M phosphoric acid containing 2 mM ascorbic acid. Approximately 1 mL of acid solution per gram of tissue was used. The sample was centrifuged at 15,000 x g for 15 minutes. The supernatant was saved and the pellet resuspended in a second volume of the acid solution. This was recentrifuged and the two supernatants combined. The combined supernatants were then filtered through a 0.22 μm filter. The filtrate was injected onto the analytical column.

RESULTS AND DISCUSSION

Voltammetry of Biopterin

Hydrodynamic voltamograms (HDV's) of the oxidation states of biopterin are shown in Figure 3. It can be seen that a potential of only +300 mV versus Ag/AgCl is necessary for the detection of tetrahydrobiopterin while a potential of +600 mV is required if 7,8-dihydrobiopterin is to be detected. For the determination of oxidized biopterin a potential of -700 mV versus the Ag/AgCl electrode is needed for reduction.

Dual-Electrode Detection

The dual-electrode amperometric detector can be used to advantage in two modes for the detection of biopterin in tissues. Both techniques employ the

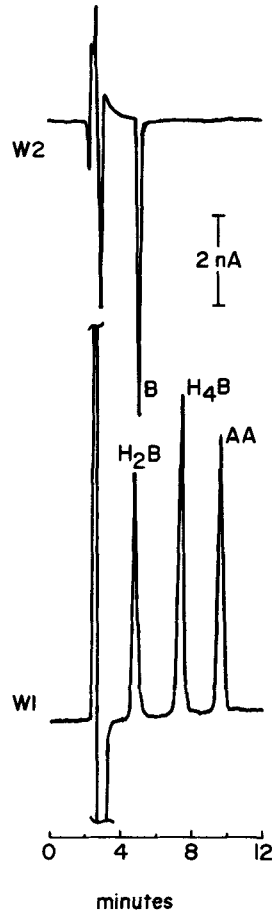


Figure 2. Standard separation of the oxidation states of biopterin. Chromatographic conditions: 3 mM octyl sodium sulfate in 0.1 M sodium phosphate buffer, pH 2.5, 15% MeOH. W1 = +600 mV, W2 = -700 mV. Peak identities: B, biopterin; H_2B, 7,8-dihydrobiopterin; H_4B, 5,6,7,8-tetrahydrobiopterin; AA,ascorbic acid.

electrodes in a parallel configuration with the electrodes adjacent to each other and normal to the direction of flow. If it is necessary to detect all oxidation states of biopterin, one electrode is poised at +600 mV to detect both reduced forms and the other electrode is poised at -700 mV to detect oxidized biopterin. However, if it is desired to detect tetrahydrobiopterin with greater selectivity and/or lower detection limits, one electrode can be operated at +300 mV to

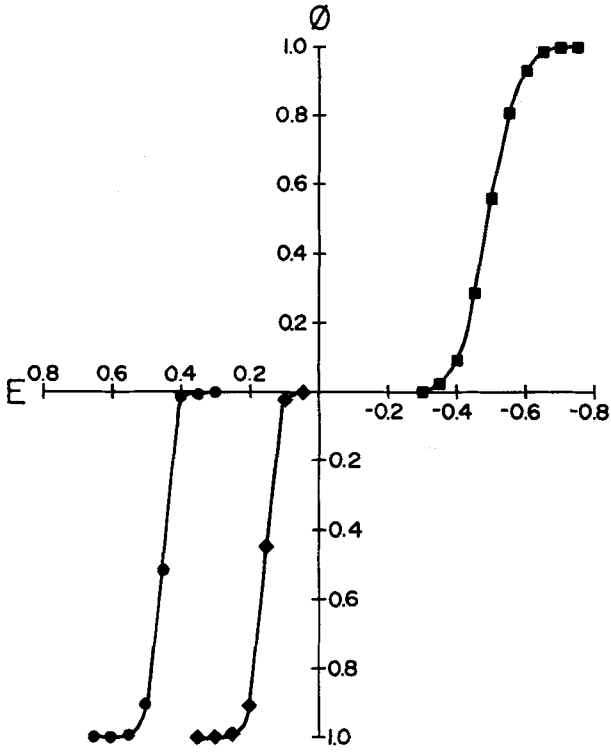


Figure 3. Hydrodynamic voltammograms of biopterin oxidation states. oxidized biopterin, tetrahydrobiopterin, dihydrobiopterin.

detect tetrahydrobiopterin and the other electrode at +700 mV to detect both tetrahydrobiopterin and 7,8-dihydrobiopterin. Figure 4 shows typical chromatograms obtained for both mouse brain and liver samples using potentials of +600 mV (lower trace) and -700 mV (upper trace). Figure 5 shows chromatograms obtained for the same samples with the electrodes operated at +600 mV (lower trace) and +300 mV (upper trace).

Linearity and Detection Limits

Response was linear over several orders of magnitude, from nanomoles to femtomoles injected, for all biopterin oxidation states. The limit-of-detection

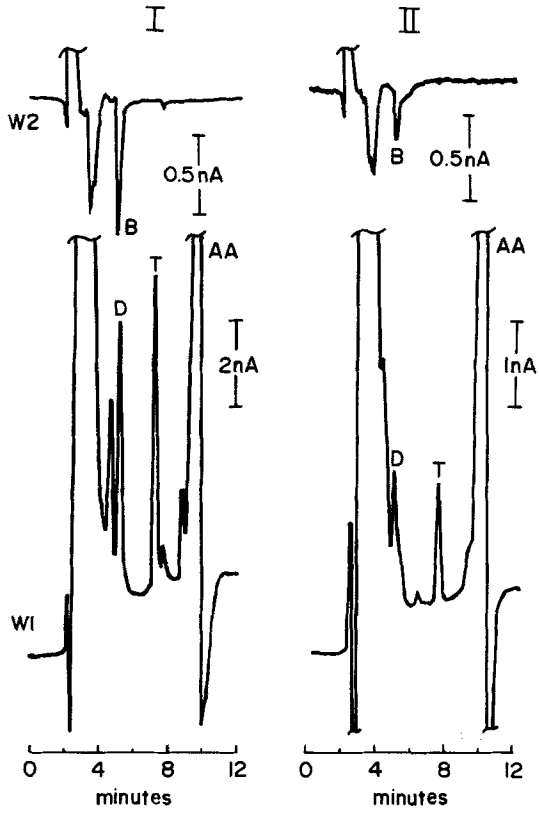


Figure 4. Detection of the oxidized and reduced forms of biopterin in mouse tissue samples. I = Liver, II = Brain. Conditions and peak identities as in Figure 2.

(S/N = 3) was 0.53 picomoles for oxidized biopterin and 0.66 picomoles for 7,8-dihydrobiopterin. The detection limit for tetrahydrobiopterin depended upon the detector potential used; at +600 mV a limit of 0.69 picomoles was found while at a potential of +300 mV a limit-of-detection of 0.53 picomoles was achieved.

Peak Identification

Peaks in the sample chromatogram were identified in two ways. First, the retention time of the sample peak was compared with the retention time of the

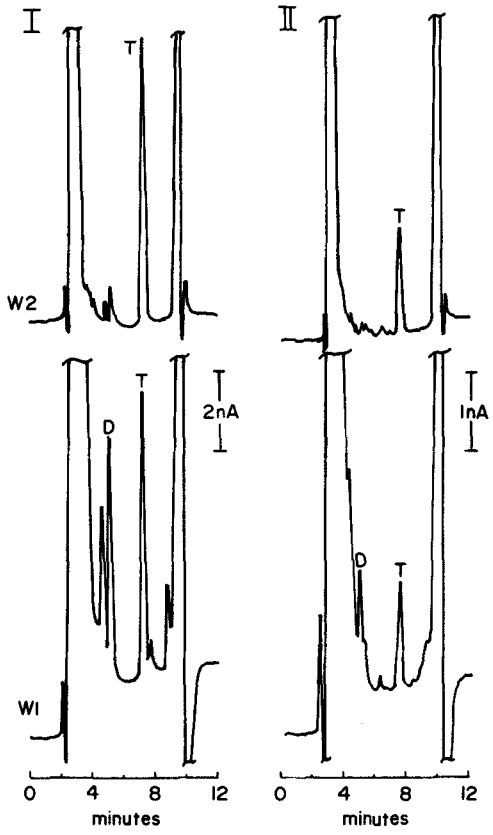


Figure 5. Selective detection of tetrahydrobiopterin in mouse tissue samples. I = Liver, II = Brain. W1 = +600 mV, W2 = +300 mV. Conditions and peak identities as in Figure 2.

authentic compound. Peaks were secondly identified by the voltammetric characterization procedure as described previously (10). Table 1 shows the voltammetric characterization data for all the oxidation states of biopterin. These two peak identification procedures allow structures to be assigned with a high degree of certainty.

Biopterin Concentrations in Mouse Tissues

As seen from Figure 4, little oxidized biopterin was detected in either the liver or brain samples. Both tetrahydrobiopterin and 7,8-dihydrobiopterin

TABLE 1

Voltammetric Characterization of Tissue Samples

DIHYDROBIOPTERIN

E(mV)	Std.[a]	Liver	Brain
+550	0.98	0.98	0.97
+500	0.68	0.68	0.66
+450	0.32	0.32	0.31
+400	0.07	0.07	0.06

TETRAHYDROBIOPTERIN

E(mV)	Std.[b]	Liver	Brain
+250	0.95	0.95	0.94
+200	0.78	0.77	0.78
+150	0.28	0.23	0.26
+100	0.06	0.07	0.06

BIOPTERIN

E(mV)	Std.[c]	Liver	Brain
-650	0.97	0.95	0.95
-600	0.91	0.89	0.88
-550	0.75	0.73	0.76
-500	0.51	0.49	0.49

[a]Current normalized to that observed at +600 mV.

[b]Current normalized to that observed at +300 mV.

[c]Current normalized to that observed at -700 mV.

TABLE 2

Biopterin Concentrations (ug/g) in Mouse Tissues

(n = 9)	H_4BIOPTERIN	H_2BIOPTERIN	BIOPTERIN
Liver	1.96	0.189	0.071
Std. Dev.	0.28	0.087	0.028
Brain	0.189	0.079	0.053
Std. Dev.	0.034	0.056	0.027

were detected in both tissues, but the prevalent form was tetrahydrobiopterin in both cases. The 7,8-dihydrobiopterin is likely derived from quinonoid dihydrobiopterin, which has been reported to be the immediate oxidation product of tetrahydrobiopterin (12). The reported half-life for the tautomerization of quinonoid dihydrobiopterin to 7,8-dihydrobiopterin being about 5 minutes (13), the detection of quinonoid dihydrobiopterin would not be expected considering the time for sample preparation. The concentrations of biopterin found in mouse tissues are listed in Table 2.

As has been shown, LCEC with dual-electrode detection offers a powerful method for the detection of the various oxidation states of biopterin in tissue samples. The ability to directly detect both the oxidized and reduced forms of biopterin is an advantage over previous methods. Dual potential monitoring also allows more selective detection of the easily oxidized tetrahydrobiopterin while still detecting the more difficult to oxidize dihydrobiopterin.

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