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ESTIMATION OF TETRAHYDRO, DIHYDRO AND FULLY OXIDISED PTERINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING SEQUENTIAL ELECTROCHEMICAL AND FLUOROMETRIC DETECTION

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SUMMARY

A method is described for the separation and detection of tetrahydro, dihydro and fully oxidised pterins in a single chromatographic run using ion-pair reversed-phase high-performance liquid chromatography. Tetrahydropterins are detected by electrochemical oxidation, dihydropterins by fluorescence following post-column electrochemical oxidation and the fully oxidised pterins by their natural fluorescence. The post-column electrochemical conversion of the non-fluorescent dihydropterins to fluorescent compounds is proportional to the amount injected over three orders of magnitude. Because of the relative selectivity of the fluorescence detection and the low potential required to oxidise the tetrahydropterins, all the oxidation species of the pterins may be measured in biological samples with minimal sample clean-up.

INTRODUCTION

The measurement of pterins by high-performance liquid chromatography (HPLC) is complicated by the fact that they exist in three oxidation states: the fully oxidised, the dihydro, and the tetrahydro forms. The fully oxidised pterins are fluorescent and can therefore be detected by this property [1]. The dihydro and tetrahydro forms are, however, virtually non-fluorescent and have in the past been measured either as total biopterin or total neopterin by fluorescence following chemical oxidation [1] or by direct electrochemical detection [2, 3]. The precolumn chemical oxidation procedure requires both acid and alkaline oxidation processes in order to determine the original oxidation state of the pterin species [1]. The electrochemical detection method requires extensive sample clean-up because of the presence in biological samples

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of other compounds which co-elute and are oxidised at the high positive potential required to oxidise the dihydropterins [2].

This paper reports the separation and detection of pterins of all three oxidation states in a single chromatographic run. This is achieved using ion-pair reversed-phase HPLC for separation and sequential coulometric electrochemical and fluorescence instruments for the detection of the pterins. The tetrahydropterins are estimated electrochemically, the dihydropterins by fluorescence following post-column electrochemical oxidation and the oxidised pterins directly by their natural fluorescence.

EXPERIMENTAL

Apparatus

HPLC was performed using a Spectra Physics SP8770 solvent delivery system, Rheodyne 7125 injector, Apex 5-μm ODS (25 cm × 4.5 mm I.D.) reversed-phase column, an ESA coulometric dual-cell electrochemical detector with the analytical electrodes set at +0.05 V and +0.4 V. The guard cell was set at +0.8 V and placed in series following the analytical electrodes. The electrochemical electrodes were followed in series by a Perkin-Elmer LS3 LC spectrofluorimeter. Excitation and emission wavelengths were set at 348 and 444 nm, respectively. A diagram of the equipment configuration is shown in Fig. 1. Peak integration was achieved using a Spectra Physics SP4270 computing integrator.

Chemicals

All the pterin species were obtained from the laboratory of Dr. B. Schircks (Buechstr. 17a CH-8645 Jona, Switzerland). Octyl sodium sulphate was purchased from Eastman-Kodak (Rochester, NY, U.S.A.).

Mobile phase

The chromatographic mobile phase was similar to that described by Lunte and Kissinger [2]. Potassium dihydrogen orthophosphate (13.61 g), octyl sodium sulphate (116 mg), EDTA (20 mg) and methanol (20 ml) were added to 800 ml of distilled water. The pH was adjusted to 2.5 with concentrated

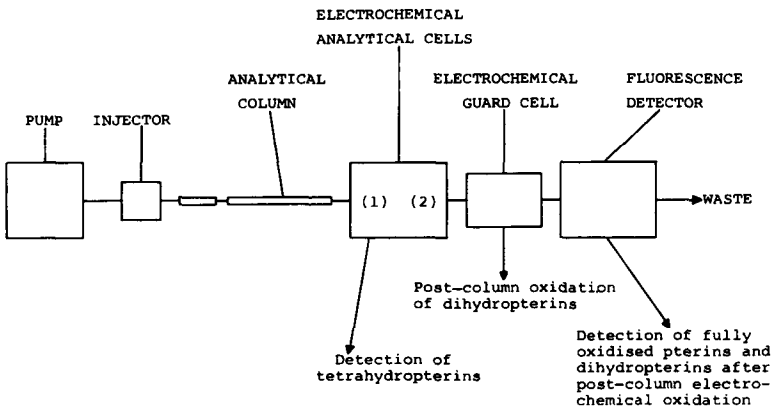

Fig. 1. Equipment configuration.

orthophosphoric acid and the final volume made up to 1 l using distilled water. The mobile phase was filtered under vacuum using a 0.45-μm aqueous filter (Millipore GVWP). Just before use dithioerythritol was added to give a concentration of 0.16 m*M*. Mobile phase was degassed with helium during the chromatographic run. The flow-rate was set at 1.3 ml/min.

Samples

Urine was passed directly into foil-wrapped vessels containing ascorbic acid (approx. 1 mg/ml) and stored at −70° C until assayed.

RESULTS AND DISCUSSION

Fig. 2 shows representative fluorescence chromatograms of an injected mixture of pterin species. Fig. 2a shows a chromatogram following post-column electrochemical oxidation in which both the oxidised and dihydropterins are detected. The oxidised pterins (neopterin, monapterin, isoxanthopterin, biopterin and pterin) are all naturally fluorescent whereas the dihydropterins (dihydroneopterin and dihydrobiopterin) have been converted to their fluorescent forms by electrochemical oxidation. This is demonstrated in Fig. 2b where the electrochemical detector has been switched off and the peaks representing the dihydropterins are greatly reduced. Tetrahydropterins are not converted to a fluorescence species following electrochemical oxidation. They can however be detected on the first analytical electrode of the electrochemical detector. Fig. 3i shows the chromatogram obtained from a standard mixture of 6*R*- (natural diastereoisomer) and 6*S*-tetrahydroneopterin, dihydroneopterin and tetrahydrobiopterin. Because of the low potential required to oxidise tetrahydrobiopterin this pterin may be measured in urine without sample clean-up [2, 3]. Fig. 3ii shows a chromatogram, produced on the first analytical electrode of the electrochemical detector, of a normal urine sample which was injected directly on to the column without pretreatment. Tetrahydrobiopterin is well separated from the other peaks. Dihydroneopterin is also oxidised at a low potential using the coulometric ESA electrochemical detector and may also be measured in urine using the first analytical electrode. 6*R*-Tetrahydroneopterin, which is well separated from dihydroneopterin using these chromatographic conditions (Fig. 3), has never been detected in urine in our laboratory. This is contrary to the results of Lunte and Kissinger [2].

Fig. 4a shows the fluorescence chromatogram obtained following post-column electrochemical oxidation of a urine specimen obtained from a patient with dihydropteridine reductase deficiency. Patients with this condition lack the enzyme which converts quinonoid dihydrobiopterin to tetrahydrobiopterin [4]. The tetrahydrobiopterin acts as a cofactor for the enzymes phenylalanine, tryptophan and tyrosine hydroxylase [5], the latter two enzymes catalysing the rate limiting steps in the biosynthetic pathways of the neurotransmitters serotonin and dopamine, respectively [5]. Quinonoid dihydrobiopterin spontaneously rearranges to 7,8-dihydrobiopterin and this peak is detected in the urine of these patients following the post-column electrochemical oxidation of this compound to the fluorescent form. As can be seen from Fig. 4b the peaks which correspond to the dihydropterins in this sample disappear when

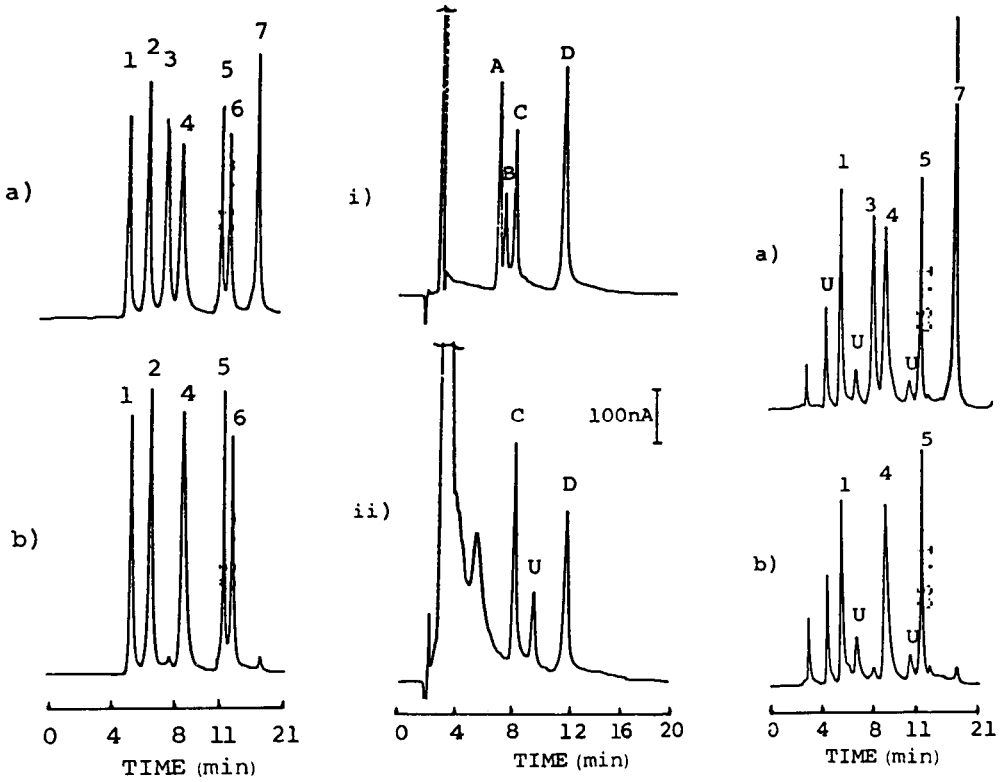

Fig. 2. Fluorescence chromatograms of (a) a mixture of neopterin (1), monapterin (2), dihydroneopterin (3), isoxanthopterin (4), biopterin (5), pterin (6) and dihydrobiopterin (7); and (b) the same mixture with the electrochemical detector switched off. Mobile phase, stationary phase and equipment configuration were as described under Experimental.

Fig. 3. Chromatograms obtained from detector one of the electrochemical detector (+0.05 V) of (i) a standard mixture of 6*R*-tetrahydroneopterin (A), 6*S*-tetrahydroneopterin (B), dihydroneopterin (C) and tetrahydrobiopterin (D); and (ii) 10 μl of urine from a healthy volunteer. Peak U, unidentified.

Fig. 4. Fluorescence chromatogram of 10 μl of urine from a patient with dihydropteridine reductase deficiency. (a) Electrochemical detector on; (b) electrochemical detector switched off. Peaks as identified in Fig. 2; U, unidentified.

the electrochemical detector is switched off. Both Hayakawa et al. [6] and Milstien et al. [7] have studied the oxidation states of the biopterins in the urine of patients with dihydropteridine reductase deficiency using the differential oxidation technique [1] and have found marked elevation of biopterin but very little dihydrobiopterin. Using the prsesent method we have detected large amounts of dihydrobiopterin in the urine of a patient with this inborn error of metabolism. It remains to be confirmed whether this is due to a patient or a methodological variation.

The ESA electrochemical detector works on a coulometric principle and hence over 99% of the sample is either oxidised or reduced depending on the positive or negative potential applied. We have found it useful to have the second detector set at +0.4 V in order to detect other compounds in the same chromatographic run. For this reason the guard cell, which would normally be

placed before the injector in order to oxidise any contaminants in the mobile phase, has been placed after the two analytical cells. The high positive potential set on the guard cell ensures that complete oxidation of any dihydropterin occurs. Fig. 5 shows the hydrodynamic voltamograms of dihydrobiopterin and dihydroneopterin and the increase in fluorescence associated with the increase in applied potential. The voltamogram for dihydroneopterin shows a two-stage oxidation profile suggesting that one electron is lost at a low potential and a second at a higher potential. It is only at the higher potential that the compound is converted to the fluorescent form. However, at the lower potential the loss of the first electron can be detected electrochemically. This is useful as an additional identity check for dihydroneopterin as it may be detected on the first electrode of the electrochemical detector (Fig. 3).

The conversion of dihydropterins to their fluorescent forms by electrochemical oxidation is proportional to the amount injected from 0 to 200 pmol; the minimum detection limits for the two dihydropterins are 0.3 pmol for dihydroneopterin and 0.54 pmol for dihydrobiopterin (signal-to-noise ratio = 3).

To demonstrate the reliability and reproducibility of the method

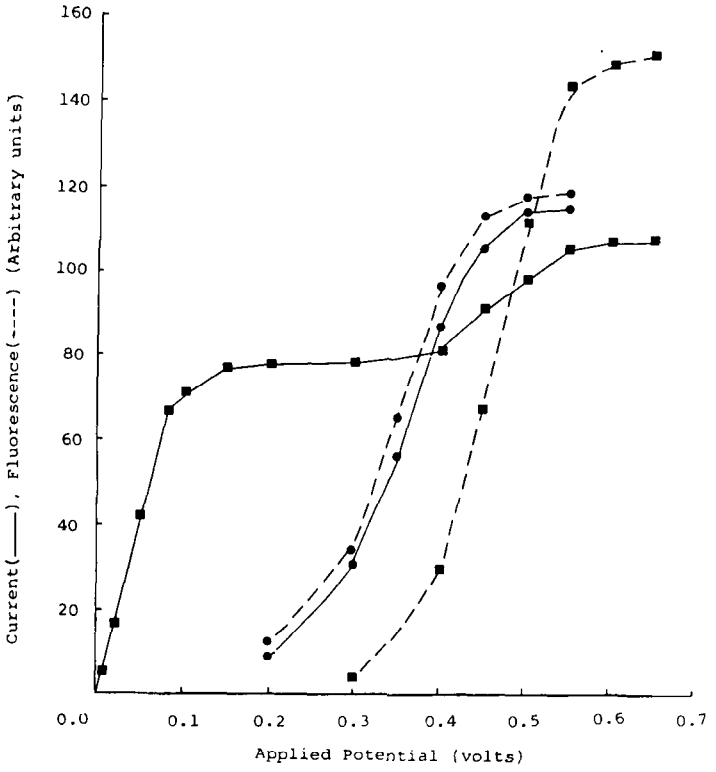

Fig. 5. Hydrodynamic voltamograms (——) and the associated fluorescence (- - -) produced following oxidation of dihydroneopterin (▪) and dihydrobiopterin (•). Hydrodynamic voltamograms were recorded from detector 1 of the electrochemical detector by adjusting the applied potential between repeat injections. Detector 2 and the guard cell of the electrochemical detector were switched off. The associated fluorescence was measured at each injection. Mobile phase, stationary phase and fluorescence settings were as described under Experimental.

TABLE I

INTRA- AND INTER-DAY COEFFICIENTS OF VARIATION OF OXIDISED, DIHYDRO, AND TETRAHYDRO PTERINS IN QUALITY CONTROL HUMAN URINE

Pterin species	Intra-day coefficient of variation (n = 6) (%)	Inter-day coefficient of variation (n = 10) (%)
Neopterin	4.45	3.96
Monapterin	4.80	11.2
Dihydroneopterin	2.97	9.41
Isoxanthopterin	4.30	13.4
Biopterin	2.15	8.45
Pterin	1.65	5.37
Dihydrobiopterin	5.54	9.22
Tetrahydrobiopterin	3.81	7.91

approximately 1000 ng/ml of all the pterins of interest were added to a sample of normal urine containing 1 ng/ml ascorbic acid. This sample was divided into aliquots and stored at −70°C. Intra-day coefficients of variation ranged from 1.7 to 5.6% and inter-day (over a period of four months) from 3.5 to 13.5% depending on the pterin species (Table I).

To date, routine methods for determining the pterins in urine have relied either on the differential iodine oxidation process [1, 6, 7] or manganese dioxide oxidation of pterins at low pH [8]. Both methods provide reliable routine procedures for the detection of abnormal pterin patterns. However, neither method directly measures the original oxidation state of the pterin species present. The present method is not presented as a routine screening procedure as the urine samples must be carefully handled to prevent spontaneous oxidation of reduced pterins, but it may provide more specific information in certain cases. The detection of dihydropterins may be of particular interest in the elucidation of the biochemical pathway leading to the formation of tetrahydrobiopterin. In atypical phenylketonuria, where there are congenital defects in the synthetic pathway leading to tetrahydrobiopterin formation [9], the ability to measure directly any dihydropterin which accumulates may help in the exact location of the metabolic block and also assist in the elucidation of the biochemical pathway if it is an unusual or unknown dihydropterin which appears.

Another method based on parallel-adjacent dual-electrode electrochemistry has been described for the detection of pterins of all oxidation states in biological samples [2]. Because of the high positive and negative potentials required to detect some of the oxidation states it was necessary to perform extensive sample clean-up procedures prior to carrying out the analysis. As a result of the relative specificity of the fluorescence detection and the ability to convert the dihydropterins to fluorescent compounds, the present method of electrochemistry and fluorescence provides a means of studying pterins of all oxidation states in urine. Preliminary studies suggest that this approach may also be suitable for the study of pterins in cerebrospinal fluid and plasma.

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