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Adsorptive stripping voltammetric analysis of some pterines in human urine*

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Abstract: The combined content of biopterin plus neopterin and the separate folic acid content of human urine have been quantitated by phase-selective alternating-current stripping voltammetry at the static mercury drop electrode. The use of a disposable C_{18} cartridge allowed the samples to be separated into two fractions, one containing biopterin and neopterin and the other folic acid. The average concentration of biopterin and neopterin together was 704 ng ml^{-1}; precision, 6.6% (RSD, $n = 7$); mean recovery, 96%. The average concentration of folic acid was 836 ng ml^{-1}; precision, 8.2% (RSD, $n = 8$); mean recovery, 91%.

Keywords: *Biopterin and neopterin assay; folic acid assay; human urine; adsorptive stripping voltammetry.*

Introduction

Pteridines (pyrazine[2,3-d]pyrimidines) have been known for nearly a century [1], although it was not until the early 1970s that their clinical relevance was appreciated, when it was observed that their concentrations in either serum or urine were altered in cancer patients and laboratory animals with experimental tumours; also, abnormalities were found in cultured tumour cells. The main pteridines found in urine are biopterin (2-amino-4-hydroxy-6-[1,2-dihydroxypropyl]pteridine; compound **I**), and neopterin (2-amino-4-hydroxy-6-[1,2,3-trihydroxypropyl]pteridine; compound **II**) in both the oxidized and reduced states [2]. Smaller amounts of xanthopterin, isoxanthopterin and sepiapterin are also present.

Biopterin is a cofactor in the hydroxylation of phenylalanine [3], tryptophan [4] and thyroxine [5], and abnormal concentrations have been reported in patients with neural diseases. Neopterin has been identified in abnormal amounts in the urine of patients with Ehrlich's tumour [6] and is considered a marker for pathological conditions that challenge the macrophage–lymphocyte-T system, such as viral infections, rheumatoid arthritis and neoplasias.

Folic acid (N-[4-[[(2-amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzoyl]-L-glutamic acid; compound **III**) is part of the vitamin B complex (vitamin B_9) and its

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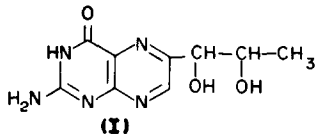
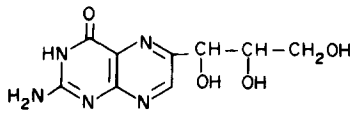


(I)



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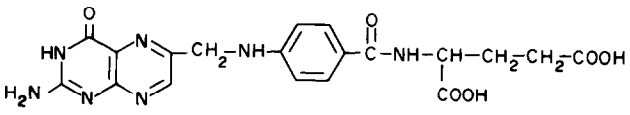

(III)

reduction products are coenzymes. Thus, tetrahydrofolic acid is important as a carrier of a formyl residue which is subsequently used in the biosynthesis of purines, serine and glycine. Folic acid is structurally related to biopterin and neopterin, since it incorporates the pterin system (2-amino-4-hydroxypteridine) in its molecule. The presence of this aromatic ring system confers exceptional adsorption onto the mercury electrode and allows preconcentration by adsorption on the surface of the electrode with subsequent measurement by stripping the adsorbed films. This technique has proved to be useful for the measurement of biologically significant molecules [10, 11].

The adsorptive stripping voltammetric behaviour of folic acid [7], biopterin and neopterin [8], and the determination of folic acid in human serum [9] have been described previously. This paper describes the assessment of biopterin plus neopterin, and separately of folic acid, in their oxidized state in human urine after a simple separation procedure.

Experimental

Apparatus

A Metrohm 663 static mercury drop electrode (SMDE) with a drop area of 0.47 mm^2 was used in conjunction with a Metrohm E506 polarograph (Herisau, Switzerland). Platinum wire was used as auxiliary electrode and all potentials were measured versus an Ag/AgCl/KCl sat. reference electrode.

Reagents

Biopterin, neopterin, folic acid and xanthopterin were purchased from Sigma (St. Louis, Missouri, USA) and used as supplied. Stock solutions (1.0×10^{-3} M) in NaOH

(0.01 M) were prepared weekly and stored in the dark at 4°C. Sodium acetate buffers (0.1 M, pH 5.0) were used as background electrolyte. All reagents were of analytical grade (Carlo Erba RPE, Milan, Italy) and water was purified in a Milli-Q (Millipore, Waters, Milford, Massachusetts, USA) system. Urine samples were from pools of human urine collected in the morning from at least 15 healthy individuals.

Procedures

Human urine samples (0.5 ml) were diluted to 10 ml with 0.1 M HAc–NaAc buffer, pH 5.0, mixed gently to avoid frothing and passed through a C_{18}-cartridge (Sep-Pak, Waters, Milford, Massachusetts, USA) previously activated by washing sequentially with 20 ml of water, 5 ml of methanol and 10 ml of the buffer solution. The effluent, together with two aliquots (of 10 ml each) of the acetate buffer passed afterwards through the cartridge, contained the biopterin and the neopterin. These were collected directly in the polarographic cell and assayed together. The materials retained on the column were eluted with 2 ml of methanol; the solvent was evaporated to dryness in a hot bath (60°C) under a stream of inert gas. The dry extract was redissolved in 20 ml of the background electrolyte by vortex mixing for 1 min at room temperature and transferred to the cell for the assessment of folic acid.

The voltammetric procedure was run in the phase-selective alternating-current (a.c.) mode in accordance with the following instrumental set-up: preconcentration time (t_p), 15 s, in open circuit and quiescent solution; superimposed alternating potential, 20 mV; frequency, 75 Hz; scan rate, 10 mV s^{-1}; sampling current time, 0.4 s. Quantitation was achieved by the standard addition method.

Solutions in the polarographic cell were de-aerated by purging with oxygen-free argon for 10 min, and for 30 s before every new scan.

Results and Discussion

All three molecules under consideration undergo a common $2e^-/2H^+$ reversible polarographic reduction process which yields the corresponding 5,8-dihydro derivative. Folic acid undergoes two more reduction processes [12, 13], while biopterin and neopterin exhibit only one more wave [14]. The first reversible process is the best to be exploited analytically, since its mechanism remains constant over a wide range of pH. Due to their structural similarity, biopterin and neopterin display very similar polarographic and voltammetric behaviour. Their peak potentials are indistinguishable and this enables their joint assessment.

Since their reduction potential (−0.500 V) was very close to that of the folic acid (−0.530 V), prior separation of the sample is necessary. Because of the polar characteristics conferred upon biopterin and neopterin by the di- and tri-hydroxypropyl substituents on C-6 they are not retained in the packing material of C_{18}-cartridges as is folic acid [9].

Preliminary experiments carried out in aqueous solutions, in which 10 ml of buffer solution was spiked with 20 µl of a 1.0×10^{-4} M solution of neopterin and passed through the cartridge, showed that partial retention of the substance takes place in the cartridge. However, subsequent washing of the cartridge with two aliquots (of 10 ml each) of buffer completely eluted the neopterin. By combining the initial eluate with the buffer, a recovery of 98% was attained for the neopterin. Conversely, no folic acid was detected when stripping voltammograms were run on either the initial eluate or the

washing fractions when a similar procedure was carried out with folic acid. The retained folic acid was eluted by methanol with a 100% recovery.

The potential-scan mode applied to the stripping of the adsorbed folic acid or biopterin and neopterin greatly affected the magnitude of their stripping peaks, and the use of a.c. resulted in the largest analytical signals when compared with those generated by differential pulse (DP) and linear sweep (LS) voltammetry. Thus, a.c. voltammetry applied to the stripping of an adsorbate of neopterin plus biopterin, formed by preconcentrating urine eluates for 30 s, equalled that obtained by DP (100 mV of superimposed voltage amplitude) after a preconcentration time of 180 s. For this reason a.c. voltammetry has been chosen for the measurements required.

The possibility of preconcentrating these substances on the mercury electrode in open circuit conditions was another way to enhance the stripping currents. A preconcentration step for folic acid in urine extracts, carried out for 30 s with stirring and in open circuit, gave rise to a current of 114 nA as opposed to a value of 63 nA obtained by operating under electrolysis at −0.3 V. Similarly, currents of 76 and 40 nA were attained for neopterin and biopterin with preconcentration from urine eluates.

The stripping voltammograms for folic acid obtained with urine extracts showed another peak at −0.380 V corresponding to an unknown matrix constituent which was retained in the C_{18}-cartridge together with the folic acid. The presence of other surfactants in the sample may pose a serious hindrance to the preconcentration of the analyte by competitive adsorption on the electrode. Figure 1 shows the evolution of the stripping peaks of folic acid in an extract of urine with increasing preconcentration times in a quiescent solution. In these conditions, as expected for semi-infinite linear diffusion [15], the peak intensity increased linearly with the half power of the preconcentration time. Conversely, forced mass convection conditions favour the adsorption of the interfering substance the peak intensity of which effectively increases to the detriment of

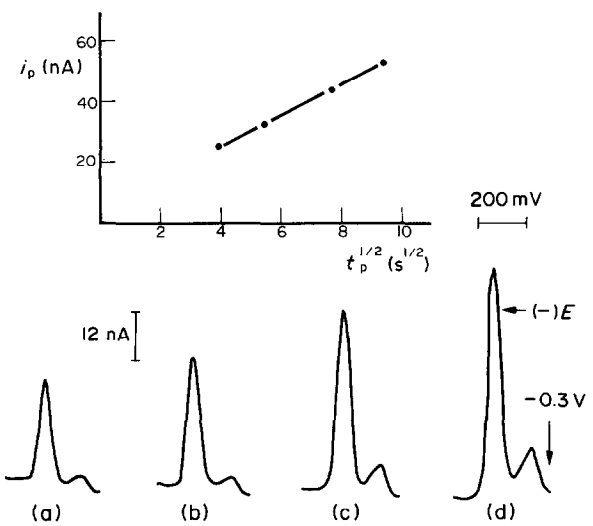

Figure 1
Effect of preconcentration time (t_p) on the a.c. stripping current of folic acid in an extract of urine. Preconcentration was carried out in a quiescent solution for: (a) 15 s; (b) 30 s; (c) 60 s; (d) 90 s. pH = 5.0; scan rate, 10 mV s^{-1}.

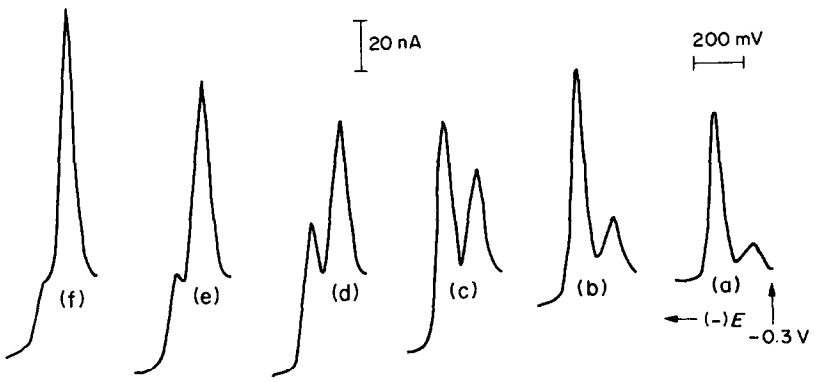

Figure 2
Effect of t_p on the a.c. stripping current of folic acid in an extract of urine. Preconcentration carried out in a stirred solution for: (a) 15 s; (b) 30 s; (c) 60 s; (d) 90 s; (e) 120 s; (f) 180 s. Stirring rate, 3000 rpm; other conditions as in Fig. 1.

the folic acid stripping signal (Fig. 2). Therefore, the preconcentration step was always carried out in unstirred solutions and in open circuit. A preconcentration time of 15 s was chosen since it provides measurable stripping signals which are unaffected by the presence of the interfering substance.

Possible interference by other pterins present in urine were considered. The addition of xanthopterin up to 10-fold the concentration of biopterin or neopterin (a ratio much higher than encountered naturally) did not show any interference.

Folic acid has a chelating tendency for metal ions [16] and a further study was prompted to ascertain whether the stripping peak is responsive solely to the free acid or also to a metal complex. Increasing amounts of EDTA added to the urine samples, giving concentrations from 10^{-6} to 10^{-2} M, did not cause any measurable variation either in the peak potential or in the stripping peak intensity for folic acid. In the same samples no effect was observed on the stripping peak corresponding to biopterin plus biopterin. Folic acid, biopterin and neopterin are, therefore, present in human urine in the free state. These results are in agreement with a recent report by Patriarche and co-workers [17] indicating that the chelating properties exhibited by folic acid are due to the pyrazine ring of the pterin moiety together with the carboxylic group of the glutamate chain, while the pterine ring alone has a very weak complexing effect. The authors also state that complexing of cadmium ions by folic acid does not occur in the presence of phosphate ions, and therefore it cannot take place in body fluids where phosphate ions are present at the same concentration.

The above analytical procedure applied to eight aliquots of pooled human urine (Fig. 3) indicated an average concentration of oxidized biopterin plus neopterin of 704 ng ml^{-1}. The overall assay precision expressed as the relative standard deviation of seven assays was 6.6% and the mean recovery was 96%. A mean concentration of 836 ng ml^{-1} was found for the folic acid, with an overall assay precision of 8.2% (RSD, $n = 8$) and a mean recovery of 91%.

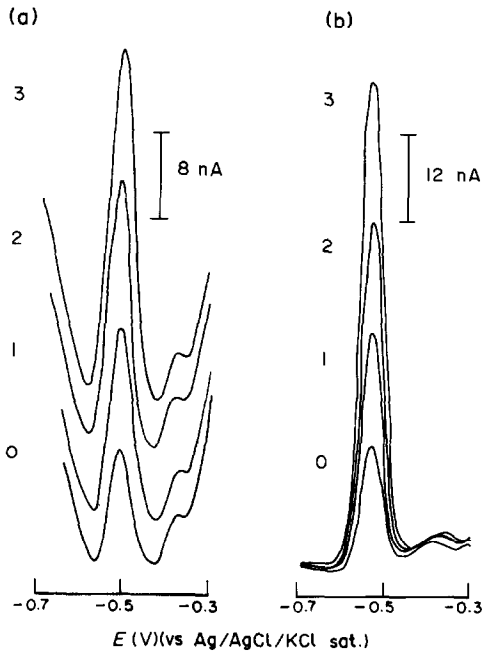

Figure 3
Quantitation of the urinary content (○) of biopterin plus neopterin (a) and of folic acid (b). Voltammograms (1)–(3) correspond to successive standard additions of 8.44 ng ml^{-1} of neopterin and 22.04 ng ml^{-1} of folic acid.

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