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DETERMINATION OF PTERINS IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON C_{18} COLUMNS CONDITIONED WITH CETYLTRIMETHYLAMMONIUM BROMIDE

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SUMMARY

A method is proposed for the determination of pterins in urine without clean-up by reversed-phase high-performance liquid chromatography on a C_{18} column previously conditioned with cetyltrimethylammonium bromide. Other endogenous compounds are retained in the column, preventing interference. Xanthopterin, neopterin, isoxanthopterin, monapterin, biopterin, 6-hydroxymethylpterin, pterin and lumazine can be determined. Retention and separation mechanisms are discussed.

INTRODUCTION

The importance of pterins in tracking diseases that involve changes in the immunological response and neurological diseases is well accepted[1,2]. The technique most often used to determine oxidized pterins in synthetic samples is reversed-phase high-performance liquid chromatography (RP-HPLC) with fluorimetric[3] detection, but there is a lack of selectivity between some pterins on different C_{18} columns and also interference from endogenous compounds in urine samples[4]. In the method described here, interference from other urine components in the determination of several pterins (I–VII) and lumazine (VIII) is minimized, so that clean-up is unnecessary, by conditioning a C_{18} column with cetyltrimethylammonium bromide (CTAB). Moreover, the selectivity of the resolution of some pterin pairs is improved. Other cationic surfactants such as benzetonium chloride gave similar results, but CTAB was chosen because it does not absorb at 235 nm, the wavelength used to measure creatinine, the compound to which measurements on analytes in urine are usually referred. The structures and pK_a values of the compounds studied are summarized in Table I.

EXPERIMENTAL

Materials

Pterins were purchased from Sigma (St. Louis, MO, U.S.A.), except monapterin, which was purchased from Fluka (Buchs, Switzerland), and were of chroma-

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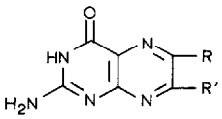
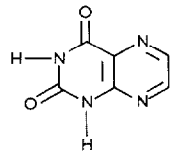
TABLE I

STRUCTURES AND pK_a VALUES[5] OF THE PTERINS AND LUMAZINE



Pterins



Lumazine

No.	*Compound*	*R'*	*R*	pK_a
I	Pterin	H	H	2.20;7.86
II	D-Neopterin	H	$CHOHCHOHCH_2OH$	2.23;7.97
III	L-Monapterin	H	$CHOHCHOHCH_2OH$	2.23;7.97
IV	Biopterin	H	$CHOHCHOHCH_3$	2.25;7.98
V	Xanthopterin	H	OH	1.6;6.3;9.23
VI	6-Hydroxymethylpterin	H	CH_2OH	–
VII	Isoxanthopterin	OH	H	−0.5;7.34;10.06
VIII	Lumazine	–	–	7.95

tographic grade. CTAB was obtained from Serva (Heidelberg, F.R.G.) and monosodium dihydrogenphosphate and disodium hydrogenphosphate from Merck (Darmstadt, F.R.G.). Water was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.). A Waters liquid chromatograph was used, consisting of the following components: a Model 590 solvent-delivery pump, a Model 420 fluorescence detector, provided with excitation filter at 365 nm and an emission band pass at 420 nm, a Model 481 variable-wavelength detector, set at 235 nm, and a Model 730 data module. A Selecta thermostated bath from Pacisa (Madrid, Spain) was used to control the column temperature in the range 14–25°C. All separations were performed on a Hypersil ODS 100 × 4 mm I.D. column from Technocroma (Barcelona, Spain).

Methods

A 5 m*M* CTAB solution was passed through the column for 16 min at a flow-rate of 1.5 ml/min (the amount of CTAB thus retained in the column was 50 mg), then 200 ml of 1.5 m*M* phosphate buffer (pH 7.2) were passed through the column to equilibrate it and prepare it for use. After 40 urine sample injections, the column was regenerated by the passage of 60 ml of methanol and reconditioning with CTAB.

The urine samples were diluted 10-fold with deionized water and filtered through a 0.45-μm filter; 10 μl of the resulting solutions were injected into the chromatograph. The peak area ratio between the fluorescent emission of pterins and the absorbance of creatinine at 235 nm was determined.

RESULTS AND DISCUSSION

Preliminary experimental work

Preliminary experiments to determine the eight pterins (Table I) in urine samples, based on experimental conditions for C_{18} columns cited in the literature, led to

several conclusions. First, their separation is impossible without a buffered mobile phase, and even then the selectivity depends significantly on the commercial C_{18} column used. Moreover, when separation is achieved and the method is applied to urine samples, interference from other endogenous compounds requires clean-up of samples. Ion-pair chromatography with a mobile phase containing anionic counter ions such as heptanesulphonate makes it necessary to work at pH 2–3 for the best selectivity, but under these conditions a significant decrease in the fluorescence quantum efficiency is observed.

When a C_{18} column was conditioned by passing cationic surfactants, such as CTAB, through it, several important effects were observed. The elution order of pterins and their separation were clearly affected. Endogenous compounds which had previously caused interference were retained on the column. In the following sections, experiments designed to optimize the separation of pterins are presented.

Effect of the amount of CTAB in the column

In order to determine the amount of CTAB retained in the column, the tubing ahead of it was first filled with CTAB solution. After connecting the column, a volume of CTAB solution was passed through it and the eluate was collected to determine its CTAB content by indirect photometry[6]. When the CTAB concentration in this eluate was lower than 0.1 m*M*, it had to be concentrated in a Sep-Pak C_{18} cartridge and eluted with a methanol–1.2 *M* hydrochloric acid (9:1, v/v) solution. The retention mechanism of CTAB must involve not only non-polar interactions, but also exchange between CTAB and silanol groups. When the amount of CTAB retained in the column (Hypersil C_{18}, 5 μm, 100 × 4 mm I.D.) was less than 50 mg, the mobile phase did not elute significant amounts of CTAB (<0.01 m*M*), so it is unnecessary to add CTAB in the mobile phase.

The effect of the amount of CTAB retained in the column on the capacity factors (k') is shown in Fig. 1. Regarding the behaviour of pterins in a conditioned column, three groups may be distinguished. The k' values of xanthopterin (pK_a = 6.3) and isoxanthopterin (pK_a = 7.4) increase with increasing amount of CTAB in the column at pH 6.5. For the non-ionized pterins two different types of behaviour were observed. First, the k' values of the four pterins containing hydroxylated carbon at position 6 (neopterin, monapterin, biopterin and 6-hydroxymethylpterin) decrease as the amount of CTAB in the column increases. This is probably due to progressive masking of free silanol groups. On the other hand, the k' values of pterin and lumazine, which do not contain hydroxylated chains, remain almost constant with variation in the amount of CTAB in the column.

Obviously, the control of CTAB retained in the column offers several practical possibilities for determining these eight compounds, depending on the conditions chosen and on the pterins present in the sample. The control of the other variables, such as pH, phosphate concentration in the mobile phase and temperature of the column, may allow optimization of the separations.

Effect of pH

The influence of pH on the k' values was studied for two different amounts of CTAB retained in the column, as shown in Figs. 2 and 3. The behaviour is similar, although small differences can be observed. The selectivity of some pairs (neopterin–

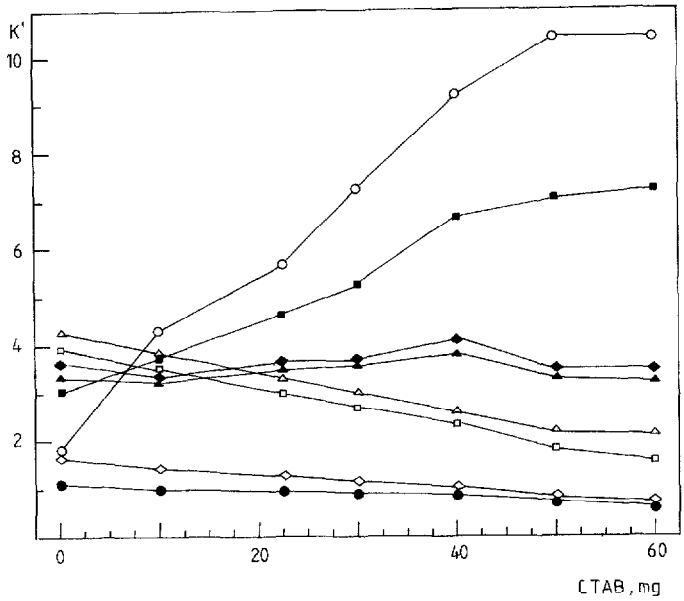


Fig. 1. Effect of the amount of CTAB in the column on the k' values of pterins. Mobile phase, 1.5 mM phosphate (pH 6.5); column, Hypersil ODS, 5 μm (100 × 4 mm I.D.). ○, Xanthopterin; ●, neopterin; ◇, monapterin; □, biopterin; ■, isoxanthopterin; △, lumazine; ◆, pterin; △, 6-hydroxymethylpterin.

monapterin and biopterin–6-hydroxymethylpterin) does not change with pH. To explain the behaviour of pterins at various pH values two main factors have to be taken into account. As the pH increases the ionization increases, causing greater ionic interaction, and hence the k' values increase. Increasing the pH, however, also in-

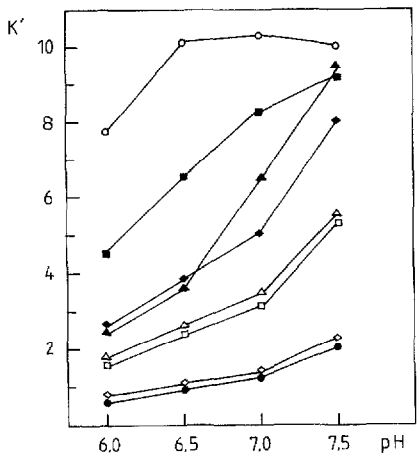


Fig. 2. Effect of pH on k' values. Amount of CTAB in the column, 50 mg; other conditions and compounds as specified in Fig. 1.

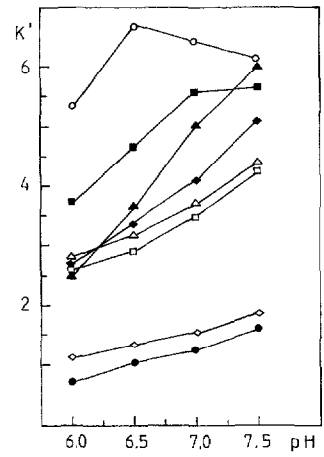


Fig. 3. Effect of pH on k' values. Amount of CTAB in the column, 20 mg; other conditions and compounds as specified in Fig. 1, except for the phosphate concentration, which was 0.5 mM.

creases the ionic strength of the mobile phase by changing the hydrogen to dihydrogenphosphate ratio, disturbing the ionic interactions, and this decreases the k' values. The combination of these two opposing effects is apparent in Figs. 2 and 3.

Effect of the phosphate concentration

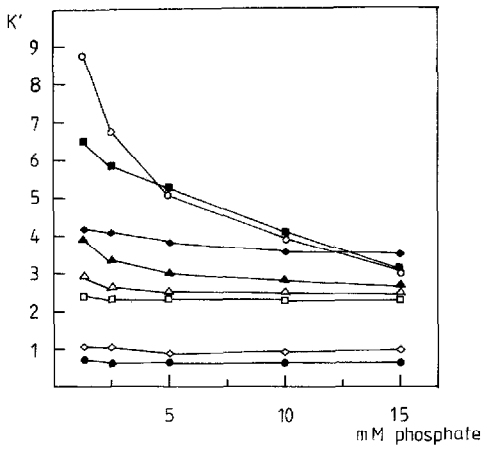
As ionic interactions are important in retention mechanisms, the ionic strength of the mobile phase must affect separations significantly. As expected, decreasing the buffer concentration increases the k' values and improves the selectivity (Fig. 4). The practical limit of reducing buffer concentration arises from difficulties in adjusting the pH in a reproducible manner. A good compromise is obtained by using a 1.5 m*M* phosphate buffer.

Effect of temperature

The column was placed in a thermostated water-bath and studies were made at different temperatures. The results (Fig. 5) show that the k' values increase slightly with decrease in temperatures, this effect being most important for 6-hydroxymethylpterin and negligible for lumazine. The selectivity of the pair biopterin–6-hydroxymethylpterin, which was difficult to improve by changing other variables, such as pH and phosphate concentration, may be adjusted by controlling the temperature.

Application to pterins in urine

Taking into account the above results, the experimental conditions chosen for the determination of pterins in urine are those specified under *Methods*. The k' values obtained for two columns with different CTAB contents are summarized in Table II. As can be seen, for the column with 50 mg of CTAB, the selectivity is satisfactory except for the neopterin–monapterin pair. In order to separate these two pterins the amount of CTAB must be limited to 20 mg, although the selectivity for separations of



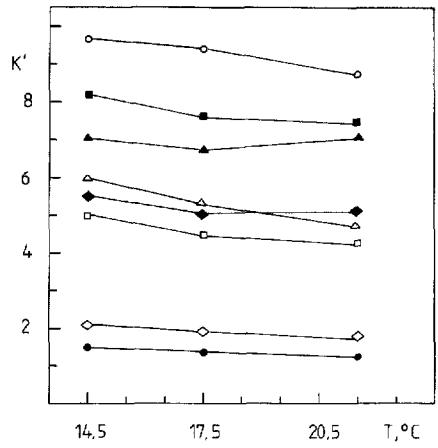


Fig. 4. Effect of phosphate concentration (pH 6.5) on k' values. Conditions and compounds as specified in Fig. 1, with 50 mg of CTAB in the column.

Fig. 5. Effect of temperature on k' values. Conditions: CTAB, 20 mg; mobile phase, 0.5 m*M* phosphate (pH 7). Pterins as in Fig. 1.

TABLE II

k' VALUES OBTAINED FOR PTERINS WITH TWO DIFFERENT AMOUNTS OF CTAB IN THE COLUMN AND TWO DIFFERENT MOBILE PHASE CONDITIONS

Conditions: (A) 22 mg of CTAB (0.5 m*M* phosphate, pH 7); (B) 50 mg of CTAB (1.5 m*M* phosphate, pH 7.2).

Compound	*k' value*	
	A	*B*
Neopterin	1.50	1.12
Monapterin	2.11	1.19
Biopterin	4.98	3.14
6-Hydroxymethylpterin	5.97	3.90
Pterin	5.59	5.70
Lumazine	7.12	6.57
Isoxanthopterin	7.12	7.90
Xanthopterin	9.64	9.90

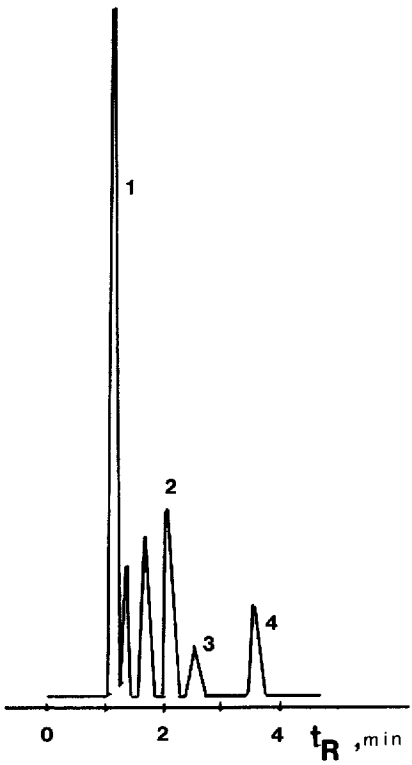


Fig. 6. Separation of some pterins in urine. Peaks: 1 = neopterin; 2 = biopterin; 3 = pterin; 4 = isoxanthopterin. For conditions, see *Methods*.

other pairs may then diminish. Presumably, these problems can be minimized by using a longer column with the same packing.

The calibration graphs were linear in the range 0.1–40 ppm. Depending on the pterin, the correlation between concentration and peak area was 2–8% and the detection limit was 0.01–0.2 ng.

Fig. 6 shows a chromatogram obtained by direct injection of a urine sample, diluted and filtered as indicated under *Methods*.

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