QUANTITATIVE DETERMINATION OF PTERINS IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

During our continuing study of pteridine metabolism, the need arose for a more rapid and quantitative determination of pterins in biological fluids. By adopting and modifying previously developed techniques, we have obtained a rapid and sensitive method that allows the simultaneous determination of eight different pterins in human urine and blood. When examined over a 10-day period, the levels of pterins excreted by a normal individual averaged the following values expressed in picomoles per mg of creatinine: biopterin, 9104; neopterin, 6018; xanthopterin, 6561; pterin, 1136; isoxanthopterin, 636; pterin-6-carboxylate, 483; and 6-hydroxymethylpterin, 315. Moreover, 6-hydroxymethylpterin and pterin-6-carboxaldehyde were detected for the first time in the blood of normal individuals.

INTRODUCTION

The rapid and quantitative measurement of pterins in urine, blood and other biological fluids has assumed a major importance because of reports describing the occurrence of elevated levels of certain pteridines in tissue culture media of malignant cells[1], in the urines[2] and in the blood of cancer patients[3], as well as in the urines of mice carrying the Ehrlich ascites tumor[4].

In order to establish the significance of such observations, and to further the study of pterin metabolism in normal as well as pathological conditions, a simple, rapid and sensitive method of analysis is required. We have recently shown that separation of all the major pterins known to occur in human urines can be accomplished by high-performance liquid chromatography (HPLC) on a strong cation-exchange column[5]. This method has also been shown to be highly sensitive, since, when coupled to fluorescence detection, it permits the detection of pterins in the picomolar range. The sensitivity of the measurements, however, is strictly dependent on the state of

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oxidation of the pterins, since they are known to yield their greatest fluorescence in the fully oxidized state. We have thus examined a variety of methods for the oxidation of reduced pterins present in urines. Since a purification step is needed before analysis by HPLC, we have modified some existing methods so that eight different pterins could be readily purified from urines and subsequently analyzed by HPLC[5].

In this communication, we also report an HPLC system for the exclusive determination of pterin-6-carboxylic acid. Finally, the method so developed was applied to the quantitative determination of unconjugated pterins excreted in the urine of a healthy subject and to the study of the diurnal variation in the excretion of these compounds.

MATERIALS AND METHODS

Collection of specimens

Urines were collected in brown-glass bottles or darkened containers to protect pterins from photo-oxidation and then frozen at $-20°$. When aliquots were needed for analysis, a specimen was melted in a water-bath at 40–45° with vigorous mixing and a 10 ml aliquot removed. Creatinine levels were determined by the method of Jaffe (as described in ref. 6) on aliquots that had been acidified to pH 1 with 6 N HCl. We found that warming of the urine to about 40° and acidification to pH 1 were necessary to obtain reproducible results. Bloods were collected in heparinized tubes and the plasma was separated from the cells by centrifugation in an International desk-top clinical centrifuge. These specimens were also stored at $-20°$ until used. All of the following operations were carried out in dim light.

Oxidation of reduced pterins

Biopterin, neopterin[7,8] and xanthopterin[9] have been reported to occur in urine as their dihydro- or tetrahydro-derivatives. The same has also been reported for biopterin present in blood[3,8]. Since our detection method strictly depends on the fluorescence efficiency of the compound, it became necessary to adopt an oxidation method that would convert the non-fluorescent hydrogenated forms of pterins into the highly fluorescent, fully oxidized forms. Three different procedures were considered and the results compared. (i) It has been reported[10] that dihydro- and tetrahydrobiopterin could be easily converted to their oxidized form by incubation of an acidic solution (0.05 N HCl) of the compounds for 20 min in a boiling water-bath. Ten different urines were thus incubated as described above after partial purification by a two-step ion-exchange procedure described later. (ii) The same set of urines were also subjected to an oxidation procedure adopted from a recently published method[8]. In short, oxidation was accomplished by addition to the acidified (pH 1) urine of a solution of 0.5 % I_2–1 % KI in 0.1 N HCl to a final concentration of 0.05 % I_2–0.1 % KI or until excess I_2 persisted in solution. We found that biological fluids differ widely from one another in the content of compounds capable of reducing I_2 to I^-. Thus, the amount of I_2–KI solution needed to obtain an excess of I_2 differed from sample to sample. Furthermore, it has been reported[8] that incubation of the sample in the presence of I_2 for 15 min would be sufficient to oxidize fully all the reduced pterins. We found this to be correct in the majority of the cases, but occasionally we encountered some urines that required a longer incubation time in the presence of I_2.

TABLE I

OXIDATION OF REDUCED PTERINS IN URINE BY I_2 AS A FUNCTION OF TIME

Aliquots (0.87 ml) of a urine were exposed to 0.05% I_2–0.1% KI (final concentration) for the length of time indicated. After each incubation, the remaining I_2 was reduced with ascorbic acid and then the samples were purified and analyzed by HPLC. Results are expressed in picomoles/mg creatinine.

Incubation time (min)	Pterins excreted (pmoles/mg creatinine)					Total output
	Xanthopterin	Neopterin[*]	Biopterin	Pt-6-CH$_2$OH	Pterin	
15	996	2000	3855	134	583	7568
30	7056	3741	10,310	247	1109	22,463
45	10,759	3963	12,069	288	1438	28,517
60	9352	3593	10,552	280	1219	24,436

[*] Neopterin levels represent the sum of both the D-erythro and the L-threo isomers.

An example is reported in Table I where four aliquots of the same urine were subjected to I_2 oxidation for different lengths of time. Clearly, in this case, an incubation for 45 min was necessary to maximize the yield of pterins that could be detected. We thus adopted a 45 min incubation time as the standard operating procedure. In order to ensure that excess I_2 was present in solution for the entire incubation period, the biological fluid being oxidized was tested periodically with starch which imparts an intense blue color to solutions containing the triiodide ion. At the end of the incubation time, the excess I_2 was removed by addition of a few drops of 1% ascorbic acid until the starch test was negative. (iii) Finally, a third method was attempted for oxidizing reduced pterins present in urines. This consisted of addition of H_2O_2 to a final concentration of 1% and incubation at room temperature for 15 min. In this case, too, the oxidation was stopped by addition of a small amount of an appropriate ascorbic acid solution. When three different aliquots of the same set of urines were oxidized in the three different ways and the results obtained were compared, we found that the I_2 and the H_2O_2 procedure gave consistently better results than the incubation in acidic medium in a boiling water-bath. The I_2 and the H_2O_2 method appeared to give consistent and comparable results. However, we adopted the I_2 method because it had already been extensively used in other laboratories and proven to be very effective in the oxidation of tetrahydro-, 7,8-dihydro- and quinoid dihydro-biopterin[8].

Purification of samples before analysis by HPLC

In order to analyze biological materials by HPLC, a preliminary purification step is often necessary[11], because these samples contain salts, compounds and high-molecular-weight molecules that would otherwise interfere with the separation as well as with the detection of the compounds of interest. Furthermore, if the purification step were omitted, the microparticulate column used for the separation in HPLC would irreversibly degenerate with a few chromatographic runs, thus making repetitive analysis of biological samples prohibitively expensive. For these reasons, all the biological materials analyzed by HPLC were purified as follows. A certain volume of the biological fluid (for urines, the volume containing 2 mg of creatinine) was first acidified to pH 1 with 6 N HCl. Then 100 μl of a 30 μM solution of internal standard were quantitatively added to the sample. The use of an internal standard is necessary

to account for any possible day-to-day variation in the purification procedure, as well as in the injection volume and in the performance of the detector. In choosing an internal standard, availability, stability, solubility, detection and non-interference with the compounds of interest were considered. We chose 6,7-dimethylpterin (6,7-DMP) as it is commercially available, highly fluorescent, and therefore easy to detect, very stable to ring oxidation and, more importantly, it chromatographed on a strong cation exchange column with a retention time longer than any other fluorescent compound present in urines. The sample to be analyzed was then subjected to an oxidation step with I_2 as described above and finally purified by a modification of a method previously described[12]. The sample is applied to a 6×20 mm Dowex 50-X8 column (H^+, 100–200 mesh), followed by 15 ml of double-distilled water. This wash is collected and analyzed for isoxanthopterin, which is not retained on the cation exchange column because of its low pK_a[13]. The Dowex 50 column is then stripped with 6 ml of 1 N NH_4OH which is directly applied to a 5×8 mm Dowex 1-X8 column (OH^-, 200–400 mesh). This column is washed with 6 ml of a solution of NH_4OH adjusted to pH 9 and then with 0.5 ml of water. Both of these washes are discarded. Finally, the Dowex 1 column is eluted successively with 2 ml of 1 N acetic acid–7% methanol–5% acetonitrile followed by 2 ml of 0.5 N HCl–7% methanol–5% acetonitrile. The following pterins are eluted in the acetic acid fraction: xanthopterin, D-erythro-neopterin, D-threo-neopterin, biopterin, pterin, pterin-6-carboxaldehyde (Pt-6-CHO), 6-hydroxymethylpterin (Pt-6-CH$_2$OH) and the internal standard, 6,7-DMP. The HCl eluate contains pterin-6-carboxylic acid (Pt-6-COOH) which binds to the anion exchange column rather strongly because of the ionizable carboxylic group. The 15 ml of water-wash containing isoxanthopterin are alkalinized with concentrated NH_4OH to pH 11, and then the entire solution is applied to another 5×8 mm Dowex 1-X8 column (OH^-, 200–400 mesh); this column is washed in the same way as described above with 6 ml NH_4OH pH 9 and 0.5 ml water, then eluted with 3 ml of 0.5 N HCl–7% methanol–5% acetonitrile. Methanol and acetonitrile were added to the eluting buffer to overcome hydrophobic interactions of the pterin ring with the Dowex backbone. The purification scheme is summarized in Fig. 1. The recovery of each pterin from urine purified by this procedure is shown in Table II. With the exception of Pt-6-CHO, all the other pterins were recovered in yields that ranged between 77% and 100%. Only 35–40% of Pt-6-CHO was recovered when added to urine and subjected to the purification procedure. Nearly 100% recovery was obtained instead when Pt-6-CHO was subjected to the purification scheme in the absence of urine. Thus Pt-6-CHO reacts with some component(s) in the urine and is not recovered quantitatively.

Blood and other biological materials were first deproteinized by addition of isopropanol to a final concentration of 70% and incubation at 4° for at least 2 h; denatured proteins were removed by centrifugation and isopropanol by extraction with six volumes of toluene. The aqueous extract was first acidified to pH 1 and then subjected to I_2 oxidation as described previously for urines; finally, the solution was purified by the same two-step ion-exchange method.

Analysis of pterins present in biological fluids by HPLC
          The acetic acid eluate from the Dowex 1 column was analyzed by HPLC on a Partisil 10-SCX (Whatman, Clifton, N.J., U.S.A.) column as described previously[5].
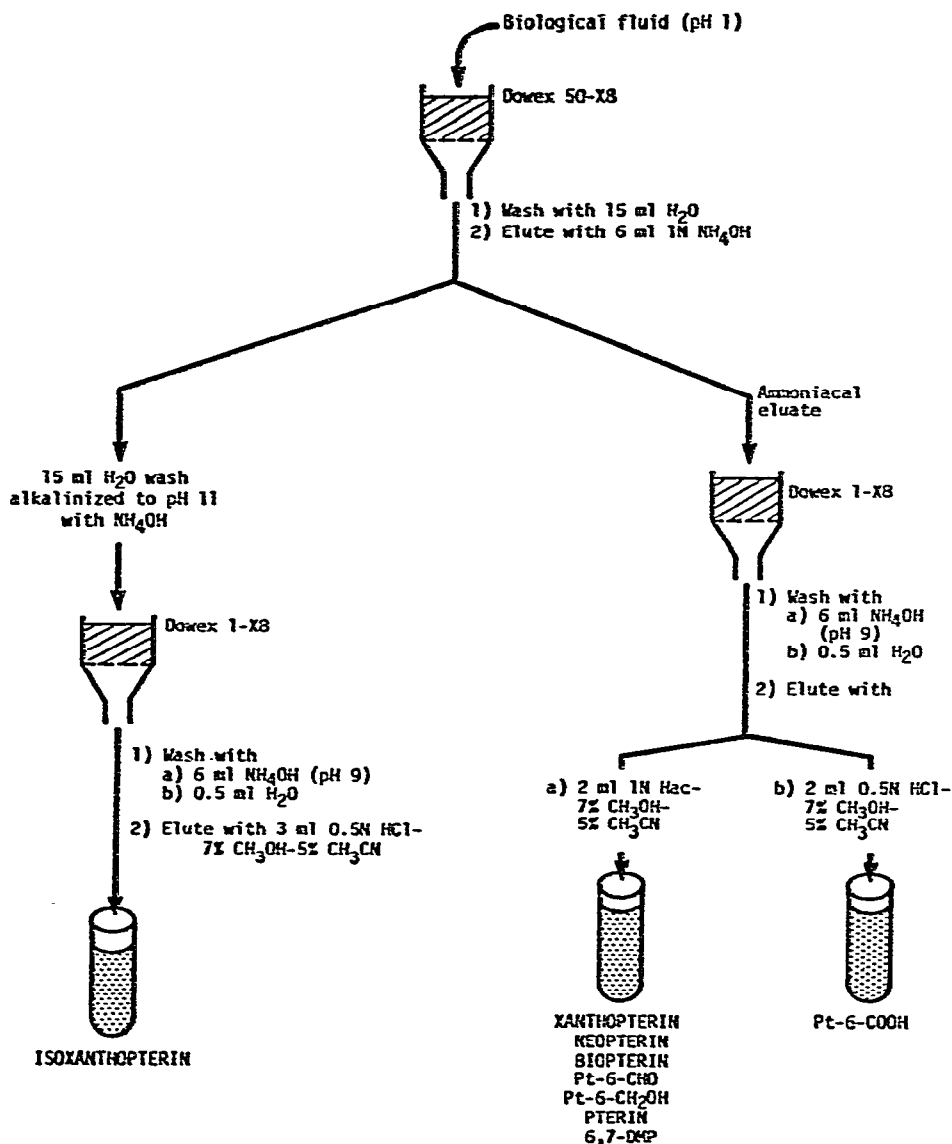
Fig. 1. Purification scheme for pterins.

Because of its exceedingly high retention time, the internal standard was eluted from the strong cation-exchange column by increasing column temperature to 55° and flow-rate to 2.5 ml/min after all the compounds of interest had been eluted at a flow of 1 ml/min and at room temperature.

The HCl eluate containing isoxanthopterin was chromatographed on two Partisol SCX columns joined in tandem, and also equipped with a 5-cm guard column. Chromatography was performed with the same solvent system used for analysis of the acetic acid eluate (1 mM NH$_4$H$_2$PO$_4$ pH 2.8–7% methanol–5% acetonitrile) at

TABLE II

RECOVERIES OF PTERINS FROM URINE PURIFIED BY THE DOWEX 50 AND DOWEX 1
PROCEDURE

A solution containing approximately 3 nmoles of each of the pterins listed below was added to a
500-μl aliquot of urine. After acidification to pH 1, the resulting solution was purified through the
Dowex 50 and 1 columns, as described in Materials and methods, and the eluates analyzed by HPLC.
Recoveries were calculated by taking the ratios of the areas under each peak obtained after purifica-
tion to those obtained when an identical solution of the same standards was injected without prior
purification.

Compound	Recovery (%)
Pt-6-CHO	40
Pt-6-COOH	77
Xanthopterin	80
Isoxanthopterin	89
Pt-6-CH$_2$OH	91
Pterin	96
Neopterin	99
Biopterin	100
6,7-DMP	100

1 ml/min and 4°. This low temperature is necessary in order to separate the iso-
xanthopterin peak from other early eluting unknown peaks which would otherwise
comigrate with isoxanthopterin at room temperature.

Finally, for analysis of Pt-6-COOH, the HCl eluate containing this compound
is first lyophilized to remove the HCl as the column used to analyze this fraction is
sensitive to the acid. The residue so obtained is dissolved in 1 ml of the same buffer
used for HPLC. Chromatographic analysis is performed in this case with a Partisil
10-SAX column (Whatman) equipped with 5-cm precolumn containing the same
packing as the main column. Isocratic elution is performed with 20 mM KH$_2$PO$_4$
(adjusted to pH 3.3 with H$_3$PO$_4$)–5% n-propanol at 1.5 ml/min and room temperature.

Detection was performed in every case with a Farrand-A4 fluorometer (Farrand
Optical, Valhalla, N.Y., U.S.A.) equipped with an excitation filter centered at 360 nm
and a narrow band emission filter at 450 nm. The aperture of the fluorometer was set
at a value of 5 and the range varied between the values of 0.3 and 10 depending on
the concentration of the samples injected.

Quantitation of pterins

Quantitative determination of pterins present in urines was accomplished by
means of linear calibration curves constructed by adding different amounts of each
pterin along with the same amount of internal standard to 0.5 ml urine aliquots.
These aliquots were subjected to the purification scheme described above (Fig. 1) and
to HPLC. The peak area ratios of each pterin peak to the 6,7-DMP peak were plotted
against the corresponding amount of standard present in the aliquot injected (100 μl).
Concentration values of xanthopterin, neopterin, biopterin, pterin, Pt-6-CHO and
Pt-6-CH$_2$OH present in urines were obtained through this type of standard curve.
An example is shown only for neopterin (Fig. 2). Pt-6-COOH and isoxanthopterin
concentrations instead were determined from linear standard curves obtained by
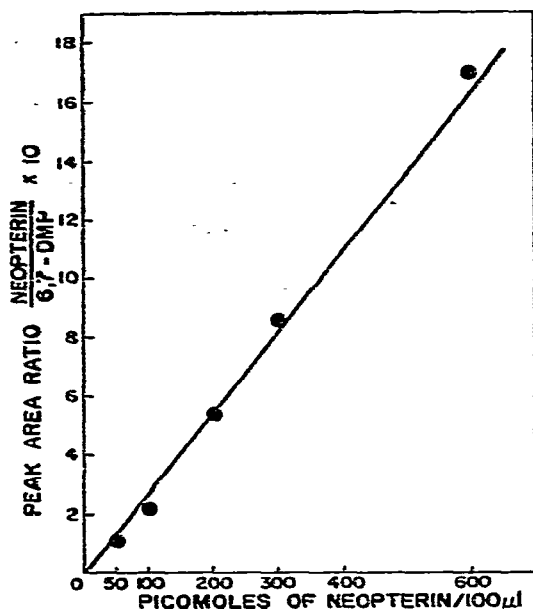
Fig. 2. Standard curve for neopterin. Increasing amounts of authentic D-*erythro*-neopterin were added to five different urine aliquots (0.5 ml) together with a constant amount of 6,7-DMP (3 nmoles). The samples were subjected to I₂ oxidation and purification by the two-step ion exchange method; then the fractions obtained by eluting the Dowex 1 columns with 1 N acetic acid–7% methanol–5% acetonitrile were analyzed by HPLC on a Partisil SCX column. Peak area ratios of neopterin to 6,7-DMP were plotted *vs.* the amount of neopterin present in the aliquot analyzed by HPLC (100 μl). Detection was performed with a Farrand A₄ fluorometer with aperture set at a value of 5 and range at 3.

simply plotting areas *vs.* the respective amount of standard present in the aliquot injected (100 μl). Area ratios of the compound under study to internal standard could not be obtained for Pt-6-COOH and isoxanthopterin since 6,7-DMP is eluted in the acetic acid fraction (see Fig. 1).

Chemicals

Standard pterins were obtained as described in ref. 5; 6,7-DMP was purchased from Regis (Morton Grove, Ill., U.S.A.). Salts used for HPLC buffers were of the highest grade available, and the organic solvents used as components of these buffers were of the HPLC grade (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.).

RESULTS

During our initial study on the excretion of unconjugated pterins in human urines, we observed that if urines were not oxidized, and no precautions were taken to keep pterins in the reduced state, increasing amounts of biopterin, neopterin and xanthopterin would be detected as a function of time when the acetic acid fraction of a purified urine was analyzed by HPLC. This indicated to us that the non-fluorescent reduced forms of those pterins were being spontaneously oxidized while

standing at room temperature in acetic acid thus becoming fluorescent and detectable at 450 nm. This confirmed previously published data on the occurrence of reduced pterins in urines[7,8]. We also found that incubation for 20 min of the acetic acid fraction containing reduced pterins in a boiling water-bath would immediately increase the level of detectable pterins. The levels obtained this way, however, were always lower than those obtained when the urine had been oxidized either by the I_2 or the H_2O_2 method before purification. Fig. 3 shows a typical chromatographic profile obtained when the acetic acid fraction of a purified urine from a normal individual was analyzed by HPLC. Peak assignments were made strictly by retention times which remain relatively constant until the column starts to deteriorate. When this happens, we found that by simply decreasing by a few degrees the temperature at which the chromatographic run is performed, we could restore the original retention times. This is possible because migration of pterins on a Partisil 10-SCX column is very sensitive to temperature[5]. It is evident from Fig. 3 that pterins occur in human urines at different concentrations, as it was necessary to change the sensitivity of the fluorometer over a ten-fold range to obtain a good signal/noise ratio for some peaks. A peak having the same mobility as authentic Pt-6-CH$_2$OH was consistently seen in all the urines examined. This compound was present in very minute amounts when compared with more abundant pterins, such as neopterin and biopterin (see Table I or III).
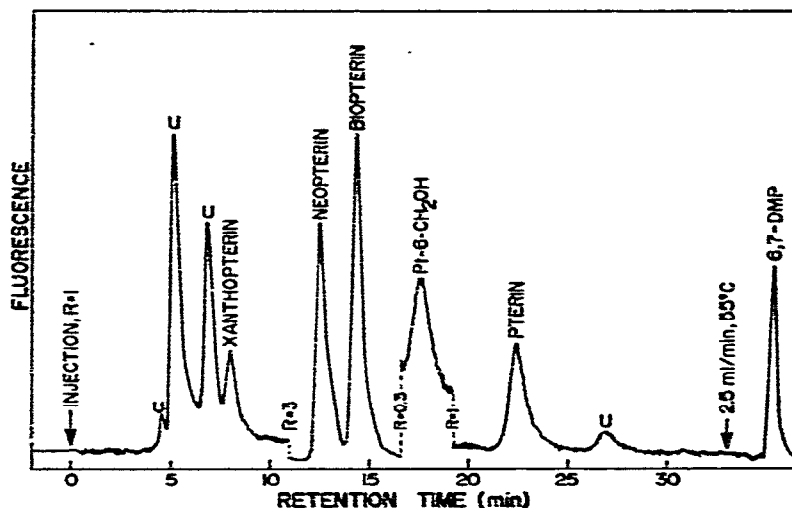

Fig. 3. Elution profile of urinary pterins in a healthy individual. The volume of urine containing 2 mg of creatinine (1 ml) was oxidized and purified as described in Methods. Chromatographic analysis of the acetic acid fraction (100 μl) was performed by HPLC on a Partisil SCX column with 1 mM NH$_4$H$_2$PO$_4$ pH 2.8–7% methanol–5% acetonitrile as the solvent at 1 ml/min and 20° for the first 32 min. Then the internal standard was eluted by placing the column in a 55° water-bath and by increasing the flow-rate to 2.5 ml/min. Detection was performed fluorometrically as described in Methods; the aperture of the fluorometer was set at a value of 5 and the range (R) varied as shown. Unidentified peaks present in the chromatogram were labeled U. The peak labeled as neopterins represents the combination of both D-*erythro*-neopterin and L-*threo*-neopterin. Because the latter is present in lower amounts than the D-*erythro*-isomer, it appears as a shoulder on the descending limb of the D-*erythro*-neopterin peak.

TABLE III

DAILY URINARY EXCRETION OF PTERINS IN A NORMAL INDIVIDUAL DURING A 10-DAY PERIOD

The volume of urine containing 2 mg of creatinine was oxidized by the I_2 method and purified as described in Methods. The different fractions obtained were analyzed by HPLC and the results expressed in picomoles/mg of creatinine.

Days	Neopterin**	Biopterin	Pt-6-CH₂OH	Pterin	Xanthopterin	Isoxanthopterin	Pt-6-COOH
1	5592	6758	264	969	6110	ND**	408
2	6370	11,448	292	1156	9073	831	372
3	5963	7414	461	855	9258	719	520
4	4848	9424	177	994	5830	ND	349
5	6370	8241	306	1312	6234	744	563
6	6630	9655	373	1139	6025	837	596
7	5852	11,138	293	1290	5376	575	562
8	8444	9828	458	1461	6357	462	612
9	5222	9690	299	1225	5796	494	510
10	4888	7448	269	956	5555	425	341

* Neopterin levels represent the sum of both the D-*erythro* and the L-*threo* isomers.
** ND = not determined.

Though only identified by its retention time on HPLC, to our knowledge, this is the first report describing the occurrence of Pt-6-CH₂OH in human urine. Its presence in very small amounts has prevented its positive identification by other means. The pterins purified from urines and present in the acetic acid fraction appear to be stable indefinitely when kept at −20° and in the dark, with the single exception of xanthopterin which slowly decreases with time over a period of several days.

The separation of peaks present in the HCl fraction containing Pt-6-COOH and the identification of this pterin are shown in Fig. 4. As can be seen from the chromatographic profile, this pterin is also present in small amounts in the urine of this normal individual. Fig. 5 depicts the chromatographic profile of the HCl fraction containing isoxanthopterin. Many other peaks were present in this and the other two fractions, but no attempts were made to identify them.

Peaks attributed to pterins were identified either directly by comigration with authentic standards or indirectly by adsorption on charcoal with concomitant disappearance of the peaks attributed to pterins, a criterion previously used by other investigators to verify the identity of certain peaks[12].

In order to gather data on the urinary levels of pterins in normal individuals and obtain clues on factors controlling their excretion, ten consecutive urines were collected daily from a young healthy adult and analyzed for their content of pterins. Table III shows the levels of pterins present in these ten urines. The mean output for each pterin as well as the maximum and minimum levels excreted are shown graphically in Fig. 6. It is clear that urinary excretion levels of pterins vary less than twofold during the 10-day period. The reason for this variation is not known and requires further investigation. However, the values reported in Table III for the individual under study are typical for people in the same age group as shown elsewhere[14]. In addition to studying the daily variation in the urinary excretion levels of pterins, we also followed excretion during a 24-h period by examining timed urines collected
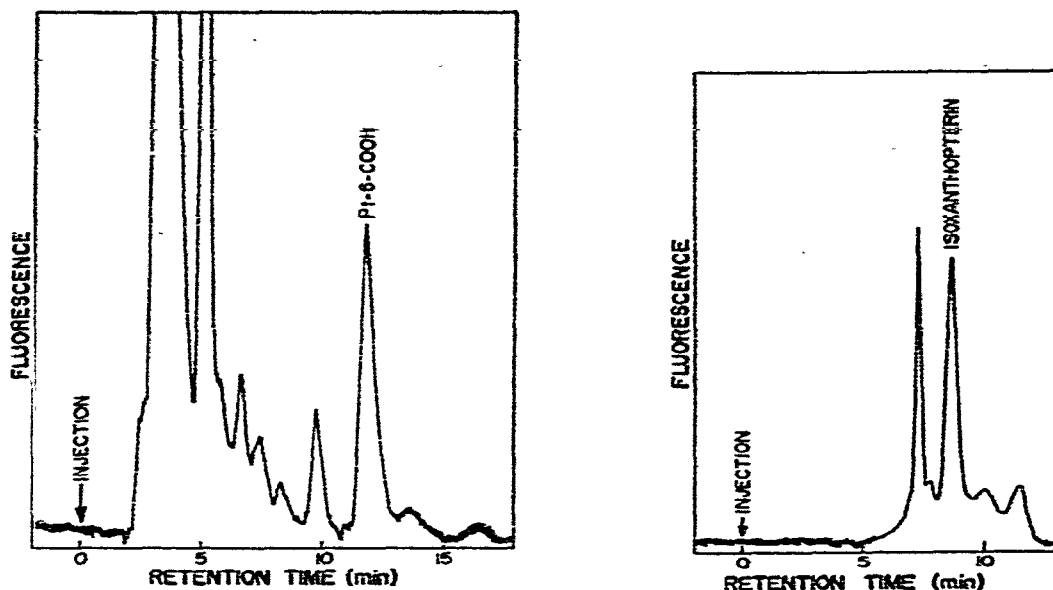
Fig. 4. Elution profile of the HCl fraction containing Pt-6-COOH from a urine of a healthy individual. The volume of urine containing 2 mg of a creatinine (1 ml) was oxidized and purified as described in the legend of Fig. 3. The HCl fraction obtained after elution with acetic acid was lyophilized to remove the HCl and the residue redissolved in 1 ml of 20 mM KH$_2$PO$_4$ pH 3.3–5% n-propanol. Then 50 μl of this solution were analyzed by HPLC on a Partisil SAX column which was eluted isocratically with the same buffer used to redissolve the residue obtained after lyophilization. Flow-rate, 1.5 ml/min; temperature, 22°. Detection was performed fluorometrically with the aperture of the fluorometer set at 5 and the range at 0.3. No attempts were made to identify all the other peaks present in this fraction.

Fig. 5. Elution profile of the HCl fraction containing isoxanthopterin from a urine of a healthy individual. The HCl eluate containing isoxanthopterin (see Fig. 1) obtained from the urine of a normal individual was analyzed by HPLC on two Partisil SCX columns joined in tandem; 100 μl were injected. Isocratic elution was performed with 1 mM NH$_4$H$_2$PO$_4$ pH 2.8–7% methanol–5% acetonitrile at 1 ml/min and in a water-bath containing melting ice (4°). Detection was performed fluorometrically as described in Methods with an aperture setting of 5 and range of 1. Unknown peaks present in the chromatogram were not identified.

every 6 h. The results of this study are shown in Fig. 7. With the possible exception of xanthopterin, all the other pterins examined were excreted in constant amounts during the 24-h period. Xanthopterin levels instead appeared to decrease between the morning and the night specimen; this change, however, is not as great as the day-to-day variation (Table III). These results validate our practice of collecting urine specimens at any time of the day, since pterins' excretion appears to remain relatively constant during a 24-h period.

    Finally, the method described in this paper can also be applied to quantitate the levels of pterins present in blood. Fig. 8 shows the typical pattern obtained for the plasma fraction of the blood of a normal individual after I$_2$ oxidation and purification as described in Methods. From the profile shown in Fig. 8, it is apparent that peaks having the same chromatographic mobility as Pt-6-CHO and Pt-6-CH$_2$OH are present in the blood of this normal individual in addition to neopterin, biopterin,
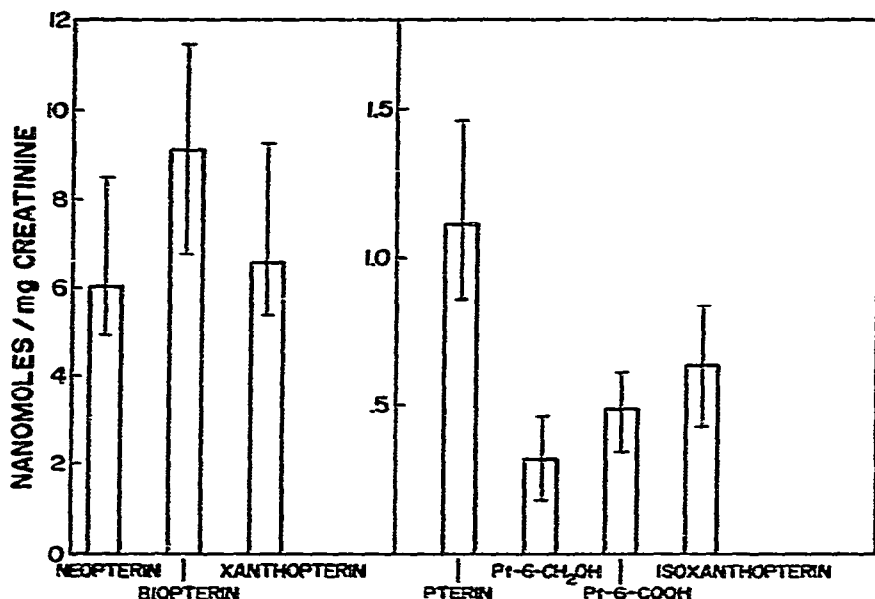
Fig. 6. Mean urinary excretion levels of pterins in a healthy individual over a 10-day period. The volume of urine containing 2 mg of creatinine was processed in each case as described in Table III. The bars indicate the average daily excretion level while the brackets indicate the lowest and the highest level excreted during the period of study. Neopterin levels represent the sum of both the D-erythro and the L-threo isomers.
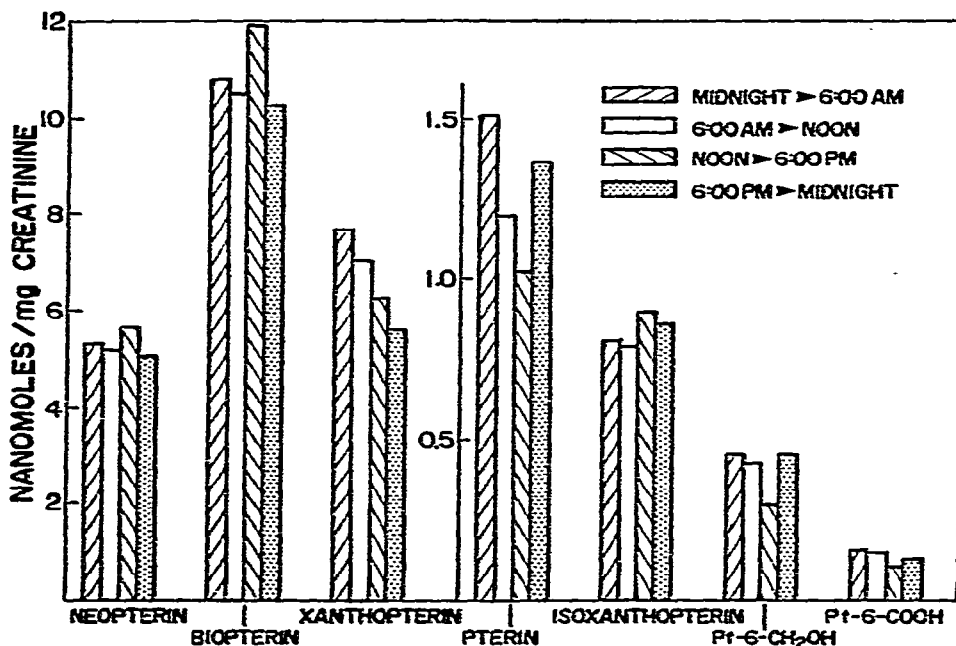

Fig. 7. Diurnal variation in the urinary excretion levels of pterins in a healthy individual. Timed urines were collected every 6 h as shown. The volume of urine equivalent to 2 mg of creatinine was processed for each specimen. Analysis was performed by HPLC as described in the legend of Figs. 3–5. Neopterin levels represent the sum of both the D-erythro and the L-threo isomers.
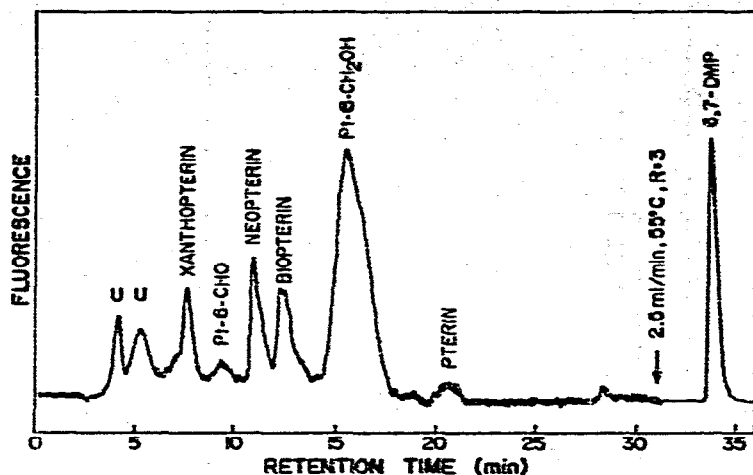
Fig. 8. Elution profile of plasma pterins of a healthy individual. Blood was collected from a healthy male individual (60 years old). The plasma was separated from the cellular fraction by centrifugation and then processed as described in Methods. After purification (Fig. 1), 300 µl of the acetic acid fraction were analyzed by HPLC in the same way described for urines in Fig. 3. The range of the fluorometer was set at a value of 0.3 and the aperture at 5. Peaks were identified by coelution with authentic standards.

xanthopterin and pterin. Moreover, the relative level of Pt-6-CH₂OH appear to be much greater in the blood than in the urine. Although it seems unlikely that other compounds could copurify and comigrate on HPLC with Pt-6-CHO or Pt-6-CH₂OH and also fluoresce at 450 nm, we feel that a more positive identification is needed for these two new pterins found in the blood of normal individuals. Pt-6-CCOH and small amounts of isoxanthopterin were also found in the plasma of the individual under study (results not shown).

DISCUSSION

The method described in this publication is a rapid, highly sensitive and relatively inexpensive technique to analyze and quantitate all the major pterins present in human urine, blood and other biological fluids. Although other rapid methods utilizing gas chromatography–mass spectrometry[10,12] or reversed-phase HPLC[15] have previously been described for the quantitation of pterins, none, however, addresses the question of the simultaneous determination of eight different pterins from a single specimen. Most of the existing methods concern themselves with the quantitation of only one or two pterins. Our experience in the detection and measurement of pterins in urines of cancer patients has shown us that quantitation of all the known pterins can be of great diagnostic importance[14]. It is our hope that because of its simplicity and reproducibility, this method will find wide application in the study of folate and pterin metabolism in the normal as well as the pathological state.

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