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HPLC determination of serum pteridine pattern as biomarkers

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ABSTRACT

Pteridinic derivatives are important biomolecules considered as biomarkers for several diseases, especially in cancer and infectious pathologies. A new fluorimetric-HPLC method for the analysis of nine pteridines in human serum has been reported. Two analytical columns composed by C18 porous and fused core particles were assayed and the results compared. Fused core particle column allows us adequate separation, in only one run and in 15 min. Acid precipitation step of the proteins and clean-up process with an Isolute ENV+ (hydroxylated polystyrene-divinylbenzene copolymer) cartridge of the serum samples have been optimized. Analytes were determined by fluorimetric detection, exciting at 272 nm and measuring the fluorescence emission at 410 nm for isoxanthopterin, at 465 nm for xanthopterin, and at 445 nm for the analysis of the other pteridines. Detection limits between 0.07 and 0.61 ng mL^{-1} were calculated according to Clayton criterium. Intraday precision varied from 1.2 to 5.3 and interday precision between 1.2 and 7.4, both expressed as RSD (%). External standard and standard addition calibrations were compared in the analysis of serum samples. The pteridine amounts in serum (expressed as ng mL^{-1} $\pm$ confidence interval) were 3.69 ± 1.78; 1.35 ± 0.24; 0.46 ± 0.14; 0.54 ± 0.24; 0.84 ± 0.55; 2.10 ± 0.51 and 0.23 ± 0.11 for XAN, NEO, MON, ISO, BIO and 6HMPT, respectively, using the external standard method. Comparable results were obtained by the standard addition method. It is noticeable that 7BIO was not detected in the healthy serum samples analyzed.

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1. Introduction

Pteridines are metabolites formed by a bicyclic pyrimidine–pyrazine moiety that occurs in a wide range of living systems and participate in relevant biological functions. Neopterin and its derivatives are synthesized in vivo from guanosine triphosphate (GTP) during the biosynthesis of 5,6,7,8-tetrahydrobiopterin (BH4), a co-factor of aromatic hydroxylation. BH4 represents the electron donor in the hydroxylation of phenylalanine to tyrosine in the liver and of tyrosine to L-dopa and tryptophan [1].

Also, it has been reported that the participation of neopterin in response to cytokines released by T-lymphocytes and natural killer cells makes this pteridine an indicator of activation of cell-mediated immunity including release by infections associated with activation of T-lymphocytes and natural killer cells, malignancies, autoimmune diseases, rejection of transplanted organs, and atherosclerosis [1,2]. Not only neopterin, but other pteridines occur everywhere in proliferating cells of different organisms; they seem to hold key positions in the metabolism of these cells [3], and there are evidences that alterations in the normal amounts of these metabolites in biologic fluids can be a consequence of several disorders. In this sense, Kaufman, in 1963 [4] informed of the importance of several pteridines in the cell metabolism of higher mammals, and it has been reported that the stimulation of T-lymphocytes in viral infections and malignant disorders causes elevation of neopterin levels in serum and urine.

Despite the diversity of pteridines with wide implications in diverse biochemistry routes of interest, the two of that have aroused the greatest interest among researchers are neopterin (NEO) and 6-biopterin (BIO) in both oxidized and reduced forms. These pteridines are analyzed in the urine of all newborns for the screening of BH4 deficiencies. Other pteridinic derivatives normally present in plasma, urine and other bodily fluids, such as xanthopterin (XAN), isoxanthopterin (ISO), pterin (PT), pterin-6-carboxylic acid (Pt6C), and 6,7-dimethylpterin also show changes in its normal concentrations in some disease states and, all of them have diagnostic value [5,6].

Hence, increased concentrations of NEO in human serum and urine have been found during allograft rejection episodes and in various malignant disorders, autoimmune diseases and viral infections, including HIV type1 [7–11]. On the other hand, a recently published study on 105 healthy children using ELISA methods established an average reference to NEO in serum of

Abbreviations: BH4, 5,6,7,8-tetrahydrobiopterin; BH2, 7,8-dihydrobiopterin; BIO, 6-biopterin; 7BIO, 7-biopterin; 6HMPT, 6-hydroximethylpterin; ISO, isoxanthopterin; MON, monapterin; NEO, neopterin; PT, pterin; PT6C, pterin-6-carboxylic acid; XAN, xanthopterin

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5.47–2.58 nmol/L and estimated as a cutoff value of 11 nmol/L [12]. In the mentioned paper the authors reported that the serum NEO concentration is lower than cutoff value in healthy children after neonatal period and it is independent of children's age or gender. The cutoff value can be compared with the amount of NEO values in different disorders and could be used as a diagnosis tool.

Also, increased NEO concentration in serum was observed in children with viral gastroenteritis. When a cut off of 11.0 nmol L^{-1} neopterin was applied, comparison yields 86.6% sensitivity and 94.3% specificity for the diagnosis of viral gastroenteritis in pediatric patients [13].

Higher levels of BIO were found in autistic children in comparison with a control group [14]. Changes in the levels of xanthopterin (XAN), BIO, pterin (PT) and 6-hydroxymethylpterin (6HMPT) were found in some cancer patients [15,16]. 7-biopterin (7BIO) is excreted by patients with carbinolaminedehydratase deficiency, using 7BIO for the differential phenylketonury diagnostic [17]. An increase in pterin-6-carboxylic acid (PT6C) and a decrease in ISO were also reported in cancer patients [18].

In this way it is proposed that the diagnostic and course of the remission of some diseases under different combination drug therapies could be determined by analyzing marker compounds in the serum or urine samples [19]. Hence, the determination of these pteridines in biological fluids seems to be of clinical relevance and it is important to establish a rapid, cheap and easy method for their determination.

For individual analysis of a selected pteridine, the ELISA method [12] has been widely used, but to analyze several pteridines HPLC techniques must be used. Although HPLC with fluorimetric detection has traditionally been the technique proposed for these analyses [20], recently and for the first time a HPLC–MS method has been proposed as a technique for the analysis of ten pteridines in the same oxidation level that they are excreted in urine [21]. Later, a HPLC–MS/MS method has also been proposed for the analysis of six pteridines in urine [22]. Also, the EC–LIF technique has been reported as an alternative technique for urinary pteridine analysis [23].

The importance of the analysis of pteridines in urine samples is well known [24,25], but the determination of these markers in serum samples is a scarce topic of research. In the bibliography, the determination of only one or two pteridines in serum or plasma with HPLC fluorimetric detection [26,27] has been described. However, sparing data are referred to for the analysis of all pteridines present in the serum samples. It was found in only a paper that determines seven pteridines in blood cell and plasma using aerial oxidation and HPLC but it spends a lot of time on each run. In the mentioned paper the last pteridine elutes in about 40 min [28]. No data about the application of EC in the analysis of pteridine mixtures in serum samples have been reported.

The direct HPLC determination of pteridines in serum is difficult compared with urine assays due, among other reasons, to the lower amounts of these compounds in a more complex matrix as serum. In this sense, the high protein contents, and the high number of potentially interfering compounds, also make the determination of these compounds [29] difficult. To eliminate the proteins present in the serum samples, the most useful method is to remove serum proteins by acid precipitation, but the potential risk of removing unknown amounts of pteridines together with the proteins has been reported [29]. However, other investigators have compared the results obtained with acid-deproteinized serum by HPLC with fluorimetric detection and serum without deproteinization with the ELISA test and similar results have been found [30,31]. To determine the total content of pteridines, the sample must be oxidized in order to convert the reduced pteridines (non-fluorescent) in oxidized forms (fluorescent). Biopterin analysis is difficult because the generation of the fully oxidized form depends strongly on the oxidation conditions. Different oxidation methods have been reported to convert BH4 in its oxidized form; air oxidation [28], I_2/I^- alkaline or acid oxidation [26], and strong potassium permanganate oxidation [32]. The most widely used method of oxidizing the biological fluids sample is by adding I_2/I^- solution in an alkaline medium. In the mentioned conditions 7,8-dihydrobiopterin (BH2) and 5,6,7,8-tetrahydrobiopterin (BH4) give rise to biopterin (BIO) [33] but the yield of BIO generated from BH4 also depends on the oxidation time and temperature. The strong dependence of the NEO/BIO ratio value, as a function of the oxidation period using I_2/I^- alkaline, has been previously reported [34]. Moreover, to reduce the presence of interfering compounds a lot of purification methods have been proposed by using different cartridges such as exchange columns [26,35]. Recently, a review with notable information about the pretreatment and determination of pteridines in biological samples has been published [36].

In the present paper an HPLC method has been developed using fluorimetric detection in order to obtain a significant serum pteridine pattern. Eight pteridines can be analyzed in a total time per run of 15 min. Two analytical columns composed of porous particular and fused core C18 particles were assayed and the results compared. Serum samples from volunteers aged between 25 and 50 years and without diagnosed pathologies were used to apply the proposed method.

2. Materials and method

2.1. Instrumentation

The chromatographic studies were performed on an Agilent Model 1260 Infinity LC high performance liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA), equipped with an online degasser (G1379A), quaternary pump (G1311A), manual six-way injection valve (Rheodyne), column oven compartment (G1316A), UV-visible diode array detector (G1315B) and rapid scan fluorescence spectrophotometer detector (G1321A). The OpenLAB LC ChemStation software (Version A.01.04) was used for controlling the instrument, data acquisition and data analysis. The chromatographic studies and analytical separation were carried out on a Zorbax-Eclipse XDB C18 (150 mm × 4.6 mm, 5.6 μm) and Poroshell 120 (150 mm × 3 mm, 2.7 μm) analytical columns. The columns were purchased from Agilent. The column temperature was set at 22 °C. The mobile phase was 2 mmol L^{-1} ammonium formate pH 7.1 in isocratic mode followed by a cleaning step with 100% ACN during 3 min. A 10 min of post-run time back to the initial mobile phase composition was used after each analysis. Mobile phases were filtered through a 0.22 μm membrane nylon filter and degassed by ultrasonication before use. The flow rate was set at 1.7 mL min^{-1} for Eclipse column and 0.4 mL min^{-1} for Poroshell column. The injection volume was 20 μL for Zorbax-Eclipse column and 10 μL for Poroshell column. As a consequence of the different excitation and emission wavelengths of the pteridines, fluorescence detector was programmed in multiemission mode, and each chromatogram was recorded simultaneously measuring the emission at 410, 445 and 465 nm, exciting in all cases at 272 nm.

A CrisonMicropH 501 pHmeter (Barcelona, Spain) equipped with a combined glass/satured calomel electrode was used for pH measurements.

Calibration curves and analytical figures of merit were performed by means of the ACOC program, developed in our research group, in MatLab code [37].

2.2. Chemicals and reagents

Pterin-6-carboxylic acid (PT6C), xanthopterin (XAN), neopterin (NEO), monapterin (MON), isoxanthopterin (ISO), pterin (PT),

6-biopterin (BIO), 7-biopterin (7BIO) and 6-hydroximethylpterin (6HMPT) were from Schircks Laboratory (Jona, Switzerland). HPLC-grade acetonitrile (ACN), HPLC-grade methanol (MEOH), hydrochloric acid and tricloroacetic acid were purchased from Panreac (Barcelona, Spain). Potassium iodide, iodine, formic acid and sodium hydroxide (NaOH) were from Sigma (Sigma-Aldrich Química, S.A., Madrid, Spain). Ammonium formate buffers were prepared by dissolving ammonium formate from Sigma in ultrapure water and fixing the pH with ammonia (Panreac). Ultrapure water was obtained from a Milli-Q system (Waters Millipore, Milford, MA, USA).

Stock standard working mixture solution of the nine pteridines (15–30 μg mL^{-1}) were prepared separately by exact weighting of each pteridine and dissolution in alkaline medium with ultrapure water and later neutralization with hydrochloric acid. These solutions were stored at −18 °C in the dark, using individual vials containing aliquots of the stock standard solution or serum sample. Each aliquot was thawed only once and they were stable for at least 3 weeks. A standard working mixture solution was daily prepared by suitable dilution of the stock analyte solutions with ultrapure water.

A pool of serum samples from twenty healthy volunteers (25–50 years old and 80% women and 20% men) obtained from members of the Research Group ANAYCO, UEx, was analyzed.

2.3. Calibration procedure

Calibration curves were performed with pteridine standard mixtures at five concentration levels as described in Table 1. Linearity was evaluated in ultrapure water in the range 0.2–7.0 ng mL^{-1}. The standards were prepared with an appropriate volume of stock standard solution of each pteridine and made up to the mark with the mobile phase. Three replicates of each concentration level were analyzed and, all of them have been previously filtered through 0.22 μm nylon filter. Calibration curves were built by plotting the peak area versus the standard concentration. The fluorescence excitation wavelength was 272 nm for all the analytes, and the emission wavelength was 410 nm for ISO, 465 nm for XAN and 445 nm for the other pteridines.

2.4. Analysis of pteridines in serum samples

An aliquot of 3 mL of fresh frozen sample serum was thawed and centrifuged at 3000 rpm for 30 min and filtered through 0.22 μm nylon filters. Then, 0.75 mL of 3 mol L^{-1} trichloroacetic acid reagent and 0.40 mL of iodine solution (2%/4% I_2/I^-) were added, and the solution was mixed thoroughly and incubated for 1.0 h at room temperature in darkness. After this, the sample was centrifuged at 3000 rpm (825*g*) for 30 min. 3.0 mL of the supernatant liquid, neutralized with sodium hydroxide, was passed through an ISOLUTE ENV+ (hydroxylated polystyrene–divinylbenzene copolymer) cartridge, previously conditioned with 5 mL of methanol and 5 mL of ultrapure water. The elution of the retained pteridines was carried out with 3.0 mL of acetonitrile:water, 80:20 (v:v). Finally, 1.0 mL of the eluate was evaporated to dryness with a stream of N_2 at room temperature, and the residue was redissolved with 0.5 mL of mobile phase. Spiked serum samples at four different concentration levels: 0.0–4.2 ng mL^{-1} for XAN; 0.0–1.2 ng mL^{-1} for ISO and 0.0–3.0 ng mL^{-1} for the rest of the analytes were analyzed with a solid-core Poroshell column. The external calibration and standard addition methods were used to calculate the concentrations of each pteridine in serum samples, and peak areas were selected as analytical signal.

3. Results and discussions

3.1. Optimization of LC separation and comparison of columns separation efficiency

With the aim to perform a simple, reliable and robust method for the efficient separation and determination of the nine pteridinic compounds in the serum samples, two reversed phase columns with different size and surface particles were tested. The columns used were: Zorbax-Eclipse XDB C18 (150 mm × 4.6 mm, 5.6 μm) and Poroshell 120 (150 mm × 3 mm, 2.7 μm). Some important parameters that influence the chromatographic

Table 1
Analytical calibration parameters.

Analyte	$t_R \pm$ SD[a] (min)	Linear range (ng mL^{-1})	Slope ± SD[a]	Determination coefficient, R^2	Linearity (%)	LOD[b] (ng mL^{-1})	LOQ[c] (ng mL^{-1})
Porous Zorbax Eclipse column							
PT6C	1.26 (±0.01)	1.0–6.0	24.2 (±0.4)	0.9974	98	0.31	1.03
XAN	2.09 (±0.04)	2.0–12.0	21.9 (±0.3)	0.9988	99	0.47	1.57
NEO	2.59 (±0.01)	1.0–6.0	35.3 (±0.6)	0.9973	98	0.32	1.07
MON	3.67 (±0.02)	1.0–6.0	44.6 (±0.6)	0.9986	99	0.23	0.77
ISO	4.48 (±0.08)	1.5–8.5	39.6 (±0.6)	0.9901	97	0.88	2.93
PT	6.76 (±0.05)	2.0–12.0	54.7 (±0.7)	0.9989	99	0.43	1.43
BIO	7.07 (±0.05)	1.0–6.0	30.4 (±0.7)	0.9961	98	0.38	1.27
7BIO	7.65 (±0.05)	1.0–6.0	20.2 (±0.5)	0.9964	98	0.34	1.13
6HMPT	8.66 (±0.06)	1.0–6.0	20.9 (±0.5)	0.9948	97	0.44	1.47
Fused core Poroshell column							
PT6C	3.07 (±0.02)	0.5–5.0	117.9 (±1.1)	0.9994	99	0.076	0.25
XAN	3.84 (±0.10)	0.7–7.0	61.8 (±2.0)	0.9921	97	0.37	1.23
NEO	4.31(±0.05)	0.5–5.0	138.7 (±0.8)	0.9989	99	0.065	0.21
MON	5.98 (±0.08)	0.4–4.0	181.5 (±1.2)	0.9996	99	0.044	0.15
ISO	7.34 (±0.22)	0.2–3.0	447.3 (±7.8)	0.9951	98	0.059	0.15
PT	10.75 (±0.17)	0.6–6.0	76.6 (±1.0)	0.9986	99	0.13	0.43
BIO	11.74 (±0.20)	0.5–5.0	99.8 (±1.7)	0.9977	98	0.14	0.47
7BIO	12.56 (±0.22)	0.5–5.0	88.9 (±0.8)	0.9991	99	0.076	0.25
6HMPT	13.70 (±0.23)	0.5–5.0	152.7 (±2.0)	0.9987	99	0.11	0.36

[a] Standard deviation, SD (n=15).
[b] Limit of detection, LOD according to Long and Winefornerd criterium [38]; $\alpha=\beta=0.05$.
[c] Limit of quantification, LOQ.
Analytical signal: peak area obtained at λ_{ex}=272 nm and λ_{em}=410, 465 and 445 nm for ISO, XAN and all other pteridines, respectively; concentration expressed as ng mL^{-1}.

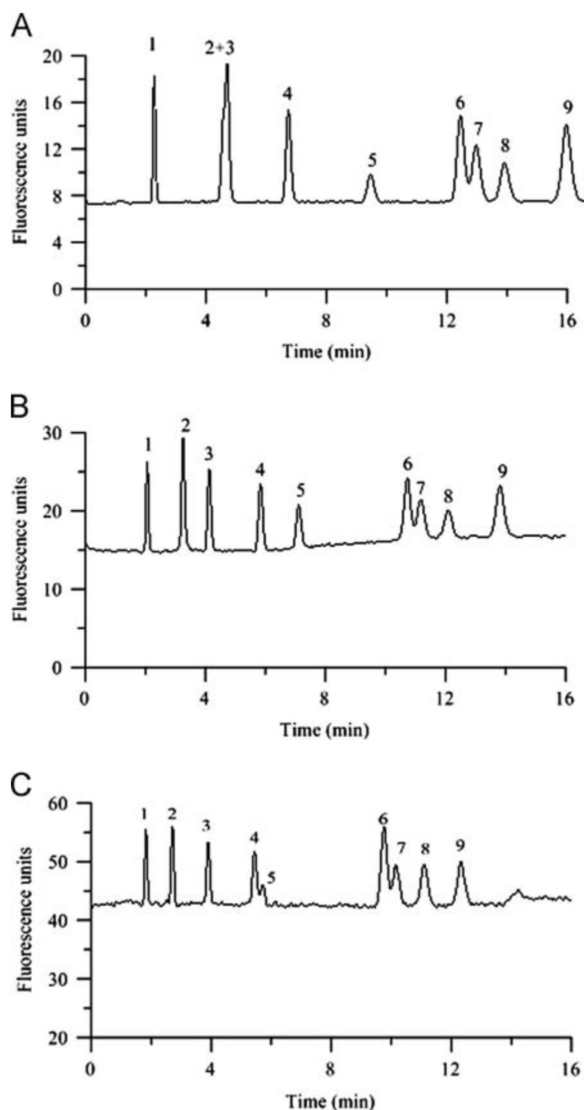

Fig. 1. Influence of the mobile phase acidity in the chromatographic separation of pteridines. pH 6.5 (A), pH 7.1(B) and pH 7.5 (C). (1) PT6C; (2) XAN; (3) NEO; (4) MON; (5) ISO; (6) PT; (7) BIO; (8) 7-BIO; and (9) 6HMPT. Conditions: Zorbax Eclipse column, flow: 1.0 mL min^{-1}, $\lambda_{ex}/\lambda_{em}$=272 nm/445 nm.

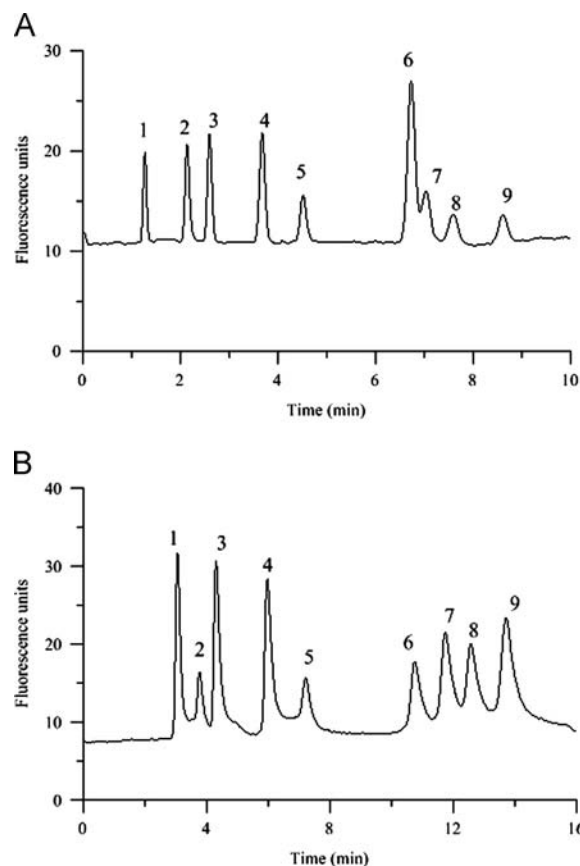

Fig. 2. Chromatograms at pH 7.1 obtained using: Zorbax Eclipse porous C18 column (flow rate: 1.7 mL min^{-1}) (A) and Poroshell fused core column (flow rate: 0.4 mL min^{-1}) (B). (1) PT6C; (2) XAN; (3) NEO; (4) MON; (5) ISO; (6) PT; (7) BIO; (8) 7-BIO; and (9) 6HMPT, Conditions: $\lambda_{ex}/\lambda_{em}$=272 nm/445 nm.

separation, such as the mobile phase composition or the flow rate, were assayed with each column.

The pH of the mobile phase has been widely studied because it is a fundamental variable in pteridinic separation. Several mobile phases composed of mixtures of aqueous solutions of formic acid and ammonium formate buffers at different pH values, were tested in both the selected columns. Retention times and resolution values were strongly affected by the acidity of the mobile phase. In both columns, an increase in the pH of the mobile phase gives rise to a diminishing of the retention time.

In Fig. 1 the chromatograms obtained with the column Zorbax-Eclipse at three pH values are plotted. At pH 6.5, XAN and NEO peaks appear overlapped, but when pH increases, the capacity factor for XAN decreases and this fact allows us an adequate resolution between these analytes at higher pH values. On the other hand, ISO is drastically affected by the pH and at pH 7.5 an overlap peak with MON can be observed. pH 7.1 provides adequate resolution of the most polar pteridines but a poor resolution between PT and BIO was observed. With this column, the flow rate was also optimized and 1.7 mL min^{-1} was the flow rate fixed for the next studies.

The influence of pH was also carried out with a Poroshell 120 column, and similar behavior of the pteridines was confirmed. It is important to emphasize that in this case and using a mobile phase of pH 7.1, the resolution between PT and BIO was improved. The optimized flow rate for this column was 0.4 mL min^{-1}.

Thus, a mobile phase composed of 2 mM ammonium formate solution at pH 7.1 was selected as optimum, and the elution was in isocratic mode with both columns.

Representative chromatograms obtained in the optimized condition with both columns are shown in Fig. 2. As can be seen, the resolution for PT and BIO was improved using a fused-core Poroshell C18 column.

3.2. Analytical characteristics of the HPLC–FSLD method for standard samples

The linearity of the method was performed by preparing standard mixtures with different concentrations depending on the column used. For the porous C18 column, the concentrations ranged between 2.0 and 12.0 ng mL^{-1} for XAN and PT, between 1.5 and 8.5 ng mL^{-1}for ISO, and between 1.0 and 6.0 ng mL^{-1} for the rest of the pteridines. As regards the Poroshell column, the linear range was from 0.7 to 7.0 ng mL^{-1} for XAN, from 0.4 to 4.0 ng mL^{-1}for MON, from 0.2 to 2.0 ng mL^{-1}for ISO, from 0.6 to 6.0 ng mL^{-1}for PT and from 0.5 to 5.0 for the other five pteridines. Emission wavelengths of 410 nm for ISO, 465 nm for XAN and 445 nm for the other pteridines were used. In all cases, the excitation wavelength was maintained at 272 nm. The standard mixtures were injected by triplicate.

The statistical calibration parameters obtained with both columns are shown in Table 1. Limits of detection (LODs) and quantification (LOQs) were calculated according to Long et al. [38]. Good linearity ($R^2 \geq 0.99$) was observed for all pteridines in both columns. Limits of detection are similar for all the compounds but as can be observed, better LODs were obtained using Poroshell column for the majority of the pteridines. These LODs were between 0.23 ng mL^{-1} for MON and 0.88 ng mL^{-1} for ISO in porous C18 column, and between 0.07 ng mL^{-1} for MON and 0.61 ng mL^{-1} for XAN in the case of Poroshell column.

3.3. Application to serum samples

Taking into account the low levels of the pteridines in the serum samples, we opted to use the Poroshell column to analyze this type of samples as the LODs found with this column are more favorable. Another aspect that we considered for using this column was that this column allows a separation to baseline of BIO, and taking into account the importance of this pteridine as biomarker analysis, we prefer this column although the run time was slightly higher.

Serum samples were prepared as described in Section 2.4. The first stage consists of the precipitation of the proteins with trichloroacetic acid and the oxidation of the non-fluorescent dihydropteridines and tetrahydropteridines to generate fluorescent pteridines. In our case, we carried out this stage using I_2/I^- as oxidative reagent, following the previous procedures [33]. In general, this treatment proved to be efficient if the analysis was carried out using the porous particle C18 column using particles of 5.6 µm. However, this treatment was not sufficient when the separation was carried out with a superficially porous microparticulate column packing; probably due to the strongly retained compounds of the serum with the subsequent blockage of the superficial particles which affect the efficiency of the column.

Exhaustive cleaning-up stages to carry out the determination of pteridines in the serum are necessary due to the complexity of the serum matrix. Thus, a clean-up stage of the oxidized serum samples with solid phase extraction was considered as a solution to this problem. In this sense, better results were obtained when ISOLUTE ENV+ (hydroxylated polystyrene-divinylbenzene copolymer) cartridges were used. Several elution mixtures composed of methanol–water and acetonitrile–water in the range of 20–80% to 100% of organic solvent, were assayed. Appropriate recovery values were obtained for 80–20 to 50–50, v/v using acetonitrile as organic solvent and, for lower amounts of acetonitrile, the recovery values notably decrease. An 80–20 (v/v) acetonitrile–water was finally selected as adequate composition for the elution of the pteridines retained in the cartridge. In these conditions, the recovery values were between 99 and 80% (3.4 to 8.6% as RSD).

Chromatograms of a fortified serum sample, once oxidation and clean-up procedure have been carried out, at λ_{ex} 272 nm and emission at 410, 445 and 465 nm are shown in Fig.3. A high increment in the ISO peak (5) at 410 nm and the XAN peak (2) at 465 nm can be observed, so these wavelengths were used for the determination of these pteridines in the serum samples.

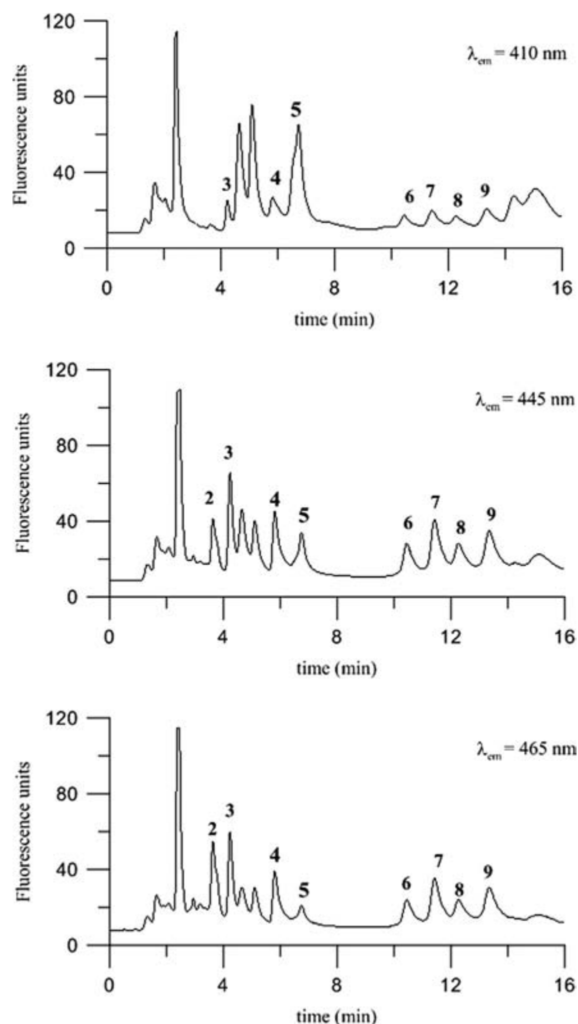

Fig. 3. Chromatograms of a fortified serum at $\lambda_{ex}/\lambda_{em}$=272 nm/410 nm (A), $\lambda_{ex}/\lambda_{em}$=272 nm/445 nm (B) and $\lambda_{ex}/\lambda_{em}$=272 nm/465 nm. Conditions: Poroshell column, flow rate: 0.4 mL min^{-1}.

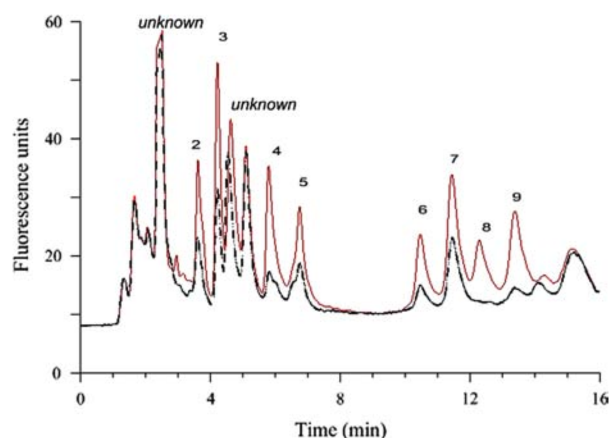

Fig. 4. Chromatograms of an oxidized serum sample (black dashed line) and a fortified oxidized serum sample (red solid line). Amount added in fortification serum: 3 ng mL^{-1} of PT6C, NEO, BIO, 7-BIO and 6HMPT; 4.2 ng mL^{-1} of XAN; 2.4 ng mL^{-1} of MON; 1.2 ng mL^{-1} of ISO and 3.5 ng mL^{-1} of PT. Conditions: Poroshell column, flow rate: 0.4 mL min^{-1}, $\lambda_{ex}/\lambda_{em}$=272 nm/445 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In Fig. 4, the chromatograms obtained for an unspiked and spiked serum samples using a fused-core Poroshell column are shown. We can observe that in the optimized conditions and in the presence of the serum, all the analytes, except PT6C, can be measured. PT6C is eluted with the solvent front jointly with other compounds, and this gives rise to an undefined signal.

Once the pretreatment of the serum samples has been optimized, chromatographic parameters such as column resolution (R), capacity factor (k') and theoretical plate number (N) were calculated using retention times and middle width of the peaks of a spiked serum sample with the objective of evaluating these parameters in the presence of the components serum. These values are summarized in Table 2, and it can be observed that k' values between 1.71 (for XAN, the pteridine less retained) and 9.02 (for 6HMPT the last eluted) have been obtained. Resolution values

Table 2
Chromatographic parameters for a spiked serum sample, using fused-core Poroshell and Zorbax Eclipse columns.

	t_R (min) ± SD	$w_{1/2}$ (min) ± SD	k' ± SD	N ± SD	R_s ± SD
Fused core Poroshell column					
t_m=1.34 ± 0.01 min					
XAN	3.63 ± 0.02	0.21 ± 0.02	1.71 ± 0.02	1609 ± 246	6.97 ± 0.33
NEO	4.23 ± 0.02	0.16 ± 0.01	2.17 ± 0.02	3682 ± 360	1.87 ± 0.07
MON	5.83 ± 0.03	0.21 ± 0.01	3.36 ± 0.03	4347 ± 476	4.99 ± 0.28
ISO	6.79 ± 0.05	0.20 ± 0.01	4.08 ± 0.05	6153 ± 214	2.74 ± 0.09
PT	10.50 ± 0.05	0.33 ± 0.01	6.85 ± 0.05	545 ± 184	8.04 ± 0.11
BIO	11.48 ± 0.05	0.36 ± 0.01	7.58 ± 0.05	5401 ± 435	1.64 ± 0.03
7BIO	12.32 ± 0.05	0.36 ± 0.01	8.21 ± 0.05	6435 ± 354	1.36 ± 0.04
6HMPT	13.40 ± 0.06	0.39 ± 0.01	9.02 ± 0.06	6523 ± 440	1.68 ± 0.04
	t_R (min) ± SD	$w_{1/2}$ (min) ± SD	k' ± SD	N ± SD	R_s ± SD
Zorbax Eclipse Column					
t_m=0.68 ± 0.01 min					
XAN	2.19 ± 0.05	0.10 ± 0.01	2.21 ± 0.079	2597 ± 519	5.87 ± 0.68
NEO	2.634 ± 0.02	0.11 ± 0.01	2.86 ± 0.02	8111 ± 2343	2.42 ± 0.24
MON	3.70 ± 0.02	0.13 ± 0.01	4.42 ± 0.04	3961 ± 158	5.01 ± 0.09
ISO	4.06 ± 0.03	0.15 ± 0.01	4.96 ± 0.05	3854 ± 80	1.48 ± 0.05
PT	6.41 ± 0.05	0.16 ± 0.01	8.39 ± 0.09	8275 ± 297	8.57 ± 0.16
BIO	6.64 ± 0.06	0.17 ± 0.01	8.73 ± 0.10	7950 ± 228	0.79 ± 0.03
7BIO	7.03 ± 0.06	0.16 ± 0.01	9.30 ± 0.12	10,350 ± 1015	1.34 ± 0.04
6HMPT	7.69 ± 0.07	0.21 ± 0.01	10.27 ± 0.12	6836 ± 486	2.02 ± 0.08

SD: Standard deviation (n=5); t_R: retention time; $w_{1/2}$: middle width peak; k': capacity factor; N: Theoretical plates numbers; R_s: resolution value

have been calculated with respect to the previous peak eluted and values between 1.36 and 8.04 were obtained. With respect to the efficacy of the column, the poorest efficacy was obtained for XAN and the best for 6HMPT.

Precision of the chromatography separation has been evaluated by injection serum samples spiked with a standard mixture of pteridines containing 4.2 ng mL^{-1} of XAN; 2.4 ng mL^{-1}of MON; 1.2 ng mL^{-1}of ISO, 3.5 ng mL^{-1}of PT and 3.0 ng mL^{-1}of the other pteridines concentration level included in the calibration range. Repeatability was evaluated analyzing the spiked serum samples, on the same day (intraday precision, n=6) and on consecutive days (interday precision, n=5). Intraday and interday precision, expressed as relative standard deviation (RSD) are between 0.72 and 5.3%, and between 1.2 and 12%, respectively.

3.4. Matrix effect

It is important to study the influence of the components present in the biological fluids, because the possible co-elution of these compounds may be the cause of the matrix effects. For this reason, and with the objective of evaluating the extension of this effect, a comparison of the slopes between external calibration curve in ultrapure water and standard addition curve from a serum sample was carried out by applying the t and F statistical tests with ACOC program, at 95% of confidence level. Statistical tests indicate that the calibration slopes for all pteridines, except for MON, were comparable. For MON, a sparing matrix suppression effect was observed.

Both external standard and standard addition methods were applied in the analysis of volunteer serum samples.

3.5. Serum samples analysis

Unfortified and fortified serum samples of healthy persons were analyzed using external standard and standard addition methods. The serum samples were treated as indicated in Section 2.4, and the obtained results for each pteridine and for both calibrations are summarized in Table 3. It is noticeable that 7BIO was not detected in any of the serum samples analyzed. P values were calculated and no statistical difference was found for 95% (P > 0.05). Confidence interval for each pteridine was also included.

Table 3
Amounts of pteridines in the serum sample using the proposed HPLC method fused-core Poroshell column.

Analito	Standard addition (n=5)		External standard (n=10)		P value[b]
	Amount (ng mL^{-1})	Confidence interval[a]	Amount (ng mL^{-1})	Confidence interval[a]	
XAN	3.69	± 1.78	3.27	± 0.40	0.360
NEO	1.35	± 0.24	1.16	± 0.11	0.060
MON	0.46	± 0.14	0.36	± 0.064	0.074
ISO	0.54	± 0.24	0.50	± 0.057	0.528
PT	0.84	± 0.55	0.81	± 0.079	0.812
BIO	2.10	± 0.51	1.85	± 0.086	0.063
7-BIO	< LOD	–	< LOD	–	–
6HMPT	0.23	± 0.11	0.15	± 0.057	0.213

[a] Calculated for α=0.05;
[b] P value for comparison between standard addition and external standard amounts. No statistical difference at 95% confidence level was found (P > 0.05).

The found results were compared with the data previously reported in the bibliography (Table 4). Because of the disparity of bibliographic units, in the mentioned table the reported contents are expressed in nmol L^{-1} for comparison, and in the original units of the reference. As it can be observed, the data about the levels of pteridines in the serum are very scarce. NEO is the pteridine that has aroused the greatest interest among researchers, and the amount found by us was 4.98 nmol L^{-1} and this value can be considered as similar to the one previously reported in healthy volunteers. On the other hand, in only one paper [28] a similar number of pteridines to the one we determined in this proposed method have been analyzed. The differences found in the Andondonskaja-renz and Zeitler method could be attributed to the treatment of the sample because an aerial oxidation process was only applied. In addition, the samples analyzed were plasma, not serum.

4. Conclusions

The separation and determination of pteridines in serum samples has been optimized using a chromatographic system coupled with sensitive and fast scanning fluorescence detection. Two columns

Table 4
Amount of pteridines in serum/plasma samples by the proposed method and bibliographic data.

Analyte	Sample	Amounts (nmol L^{-1})	Amount (expressed as in Reference)	Reference
NEO	Healthy volunteers (serum)		Idem	[1]
	Age 1–18	3.5–13.5		
	19–75	2.6–8.7		
NEO	Healthy children (serum)	2.87–14.78	Idem	[12]
		Mean: 5.47 ± 2.58		
		Cut off: 11		
NEO	Healthy volunteers (serum)		Idem	[11]
	Age < 18	6.8 ± 3.6		
	19–75	5.3 ± 2.7		
NEO	Healthy children (serum)	2.88–14.8	Idem	[13]
		Mean: 4.73		
	Viral gastroenteritis Children (serum)	6.03–171		
		Mean: 23.7		
NEO	Healthy adults (serum)	4.82	Idem	[29]
BIO_{total}	Healthy adults (plasma)	23.5 ± 1.3	Idem	[26]
BH4		14.8 ± 1.6		
NEO	Healthy adults (serum, I_2/I^- oxidation)	21.8	5.52 μg L^{-1}	[27]
BIO_{total}		6.91	1.64	
NEO	Healthy adults (plasma, aerial oxidation)	8.26	2092	[28]
XAN		1.74	312	
MON		0.17	42	
ISO		0.70	126 pg mL^{-1}	
BIO		3.04	720	
6HMPT		0.87	168	
PT		6.78	1106	
NEO	Healthy adults (serum, I_2/I^- oxidation)	4.98	1.26	Proposed method[a]
XAN		19.43	3.49	
MON		1.61	0.41	
ISO		2.90	0.52 ng mL^{-1}	
BIO		8.30	1.97	
6HMPT		0.98	0.19	
PT		5.08	0.83	
7BIO		< LOD	< LOD	

[a] Mean value calculated between those obtained by the external standard and standard addition methods.

were assayed and, due to the better resolution obtained between PT and BIO peaks and, considering the notable importance as marker of BIO in the biological fluids, the Poroshell column was selected. On the other hand, not significant differences were found by external standard and standard additions procedures in the levels of the pteridines in the serum samples analyzed. This allows us the analysis of all pteridines using the external standard method in only one run. The proposed methods permit us to carry out, for the first time, the determination of eight pteridines present in the serum, previously oxidized by the established I_2/I^- method, and to obtain the pattern of these pteridines in the serum samples of healthy persons. In general, the levels found for each pteridine are not very different from those reported for other researches.

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