

Effect of pterin impurities on the fluorescence and photochemistry of commercial folic acid

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ABSTRACT

Folic acid, or pteroyl-L-glutamic acid (PteGlu) is a conjugated pterin derivative that is used in dietary supplementation as a source of folates, a group of compounds essential for a variety of physiological functions in humans. Photochemistry of PteGlu is important because folates are not synthesized by mammals, undergo photodegradation and their deficiency is related to many diseases. We have demonstrated that usual commercial PteGlu is unpurified with the unconjugated oxidized pterins 6-formylpterin (Fop) and 6-carboxypterin (Cap). These compounds are in such low amounts that a normal chromatographic control would not detect any pterinic contamination. However, the fluorescence of PteGlu solutions is due to the emission of Fop and Cap and the contribution of the PteGlu emission, much lower, is negligible. This is because the fluorescence quantum yield (Φ_F) of PteGlu is extremely weak compared to the Φ_F of Fop and Cap. Likewise, the PteGlu photodegradation upon UV-A radiation is an oxidation photosensitized by oxidized unconjugated pterins present in the solution, and not a process initiated by the direct absorption of photons by PteGlu. In brief, the fluorescence and photochemical properties of PteGlu solutions, prepared using commercially available solids, are due to their unconjugated pterins impurities and not to PteGlu itself. This fact calls into question many reported studies on fluorescence and photooxidation of this compound.

1. Introduction

Folic acid, or pteroyl-L-glutamic acid (PteGlu) is a conjugated pterin derivative, with a chemical structure composed by three moieties: a 6-methylpterin (Mep) residue, a *p*-aminobenzoic acid (PABA) residue, and a glutamic acid (Glu) residue (Scheme 1a). In living systems, PteGlu is present in multiple forms including molecules attached to several glutamate residues and dihydro and tetrahydro derivatives. Folate is the generic term for this large family of chemically related compounds.

Tetrahydrofolate and its derivatives act as coenzymes in one-carbon transfer reactions required in the biosynthesis of nucleic acids and proteins [1] and, in consequence, these compounds are essential for a variety of physiological functions in humans. Moreover, a deficit of folate leads to several diseases such as megaloblastic anemia [2], coronary heart disease [3], neurological disorders [4], and fertility

problems [5–7]. Folate requirements increase in periods of rapid cell division and growth, and, therefore, it is important that pregnant women keep folate concentrations at an appropriate level [8]. Folate deficiency in pregnancy is related to neural tube defects (NTD), such as spina bifida and anencephaly [9,10].

Mammals do not synthesize folates and therefore they have to get them from food. In some situations, like anemia or pregnancy, folate supplementation is needed. PteGlu is inexpensive to produce, more stable than most members of folate's family and efficiently metabolized into biologically active derivatives, such as 5-methyltetrahydrofolic acid. Due to these properties, PteGlu is used in tablet form and in fortified foods for dietary supplementation [1,11].

The correlation between NTD and UV-A (315–400 nm) exposure has been described in amphibian larvae [12] and in women who had used artificial tanning sun beds during the first weeks of pregnancy [13]. In addition, epidemiological data have shown that the prevalence of NTD

Abbreviations: absorbance, A; absorbed photon flux density, q_n , $\mu\text{mol m}^{-2}\text{s}^{-1}$; acetonitrile, ACN; ammonium acetate, NH_4Ac ; 6-carboxypterin, Cap; emission wavelength, λ_{em} ; excitation wavelength, λ_{exc} ; folic acid, PteGlu; folic acid radical cation, $\text{PteGlu}^{\cdot+}$; formic acid, HCOOH ; 6-formylpterin, Fop; fluorescence, FL; fluorescence quantum yields, Φ_F ; glutamic acid, Glu; high-performance liquid chromatography, HPLC; hydrogen peroxide, H_2O_2 ; incident photon flux density, q_n , $\mu\text{mol m}^{-2}\text{s}^{-1}$; incident photons per time interval, q_n , $\mu\text{mol m}^{-2}$; liquid chromatography/mass spectrometry, LC/MS; 6-methylpterin, Mep; neural tube defects, NTD; *p*-aminobenzoic acid, PABA; *p*-aminobenzoylglutamic acid, PABA-Glu; photosensitizers, Sens; photosensitizer radical anion, $\text{Sens}^{\cdot-}$; reference fluorophore, R; retention times, t_R ; superoxide anion, $\text{O}_2^{\cdot-}$; total fluorescence intensities, I_F ; triplet excited state of the sensitizer, $^3\text{Sens}^*$; volume, V

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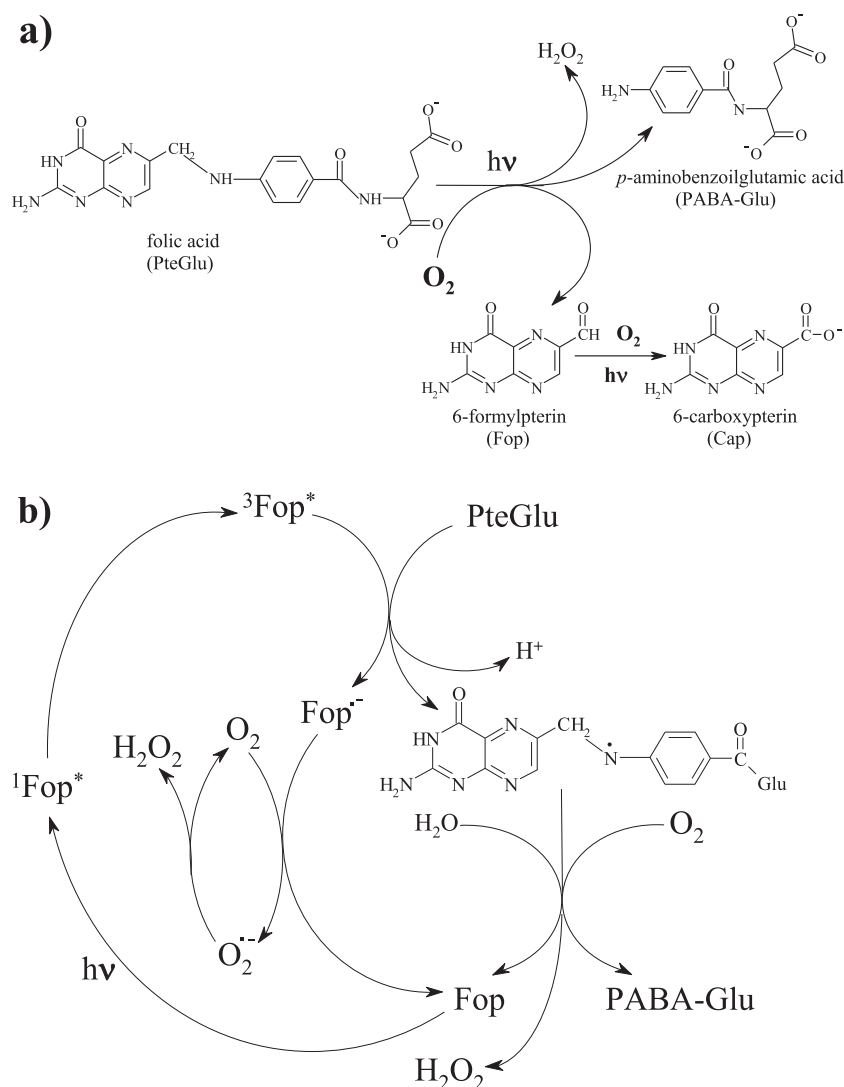
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Scheme 1. a) Photooxidation of PteGlu in air-equilibrated aqueous solutions under UV-A irradiation. b) Mechanism of the oxidation of PteGlu photosensitized by Fop. Similar processes take place with other aromatic pterins acting as photosensitizers.

is higher in light-skinned people [14,15]. In 1978, Branda and Eaton proposed that one of the main functions of skin pigmentation is to avoid photolysis of folate [16]. This hypothesis was based on the following facts: i) light-skinned patients undergoing photochemotherapy (*i.e.*, psoralen plus UV-A) showed lower serum folate levels than healthy controls, and ii) a 30–50% loss of folate in human plasma was observed after *in vitro* exposure to simulated sunlight. Recent reports indicate that both *in vitro* and *in vivo* exposure of human blood to UV-A radiation, lead to photodegradation of folate [17,18].

PteGlu can be excited by solar radiation and artificial sources of light since its absorption spectrum shows bands in the UV-B (280–315 nm) and UV-A spectral regions (Fig. S1, Supplementary material). The photodegradation of PteGlu was reported for the first time in the late 1940s [19] and, since then, many studies on the photochemistry of this compound, as a model of folates, have been conducted in aqueous solutions and other media [20–28]. In the absence of oxygen, PteGlu is photostable, but excitation in air-equilibrated solutions leads to cleavage and oxidation of the molecule, yielding 6-formylpterin (Fop) and *p*-aminobenzoilglutamic acid (PABA-Glu) as photoproducts (Scheme 1a). In turn, Fop is transformed into 6-carboxypterin (Cap) upon further photooxidation.

Upon UV-A irradiation the degradation of PteGlu in the presence of oxygen is a sigmoidal function [22,24–26]: after a period of time the

rate of PteGlu consumption significantly increases. In 2010, we reported that the photooxidation products of PteGlu are able to photoinduce the degradation of this molecule (Scheme 1b) [29]. That means that an “auto-photo-catalytic” effect is involved, where Fop photosensitizes the oxidation of PteGlu, and that explains the acceleration in the consumption of reactant when its products accumulate in the medium. In general terms, the photosensitization consists in the chemical alteration occurring in one molecular entity as a result of the initial absorption of radiation by another molecular entity called the photosensitizer. This process, in which no excitation of PteGlu is needed, also takes place with other pterins as photosensitizers (Sens), thus revealing a general mechanism. After excitation of the Sens, the first step of the process involves an electron transfer from the PABA unit of PteGlu to the triplet excited state of the Sens ($^3\text{Sens}^*$) to form the corresponding radical ions, the photosensitizer radical anion ($\text{Sens}^{\cdot-}$) and the PteGlu radical cation ($\text{PteGlu}^{\cdot+}$). The electron transfer from $\text{Sens}^{\cdot-}$ to O_2 regenerates the Sens and forms superoxide anion ($\text{O}_2^{\cdot-}$), which disproportionates to form hydrogen peroxide (H_2O_2). Finally, PteGlu degradation occurs as a result of the trapping of $\text{PteGlu}^{\cdot+}$ by oxygen (Scheme 1b).

Although a large amount of reports about photodegradation of PteGlu under UV-A radiation have been published, many questions about the photochemistry of this compound remain without answer.

What is the mechanism of the initial step, that is, before the photosensitized process dominates the degradation of PteGlu? Unconjugated pterins are present as impurities in the commercial presentation of folates? If so, does the photosensitization by these compounds contribute to the photodegradation of PteGlu? When the photophysical properties, such as the fluorescence, of PteGlu are studied, do the unconjugated pterins, present as impurities or generated photochemically, contribute?

Motivated by the importance of the photochemistry of folates *in vivo*, this work was aimed to answer the questions raised in the previous paragraph. For this purpose, aqueous solutions of PteGlu were prepared using different commercially available solids and were analyzed by high-performance liquid chromatography (HPLC) and mass spectrometry to investigate pterinic impurities. PteGlu was purified by HPLC and the photochemical and spectroscopic properties of solutions of purified and unpurified PteGlu were compared.

2. Materials and Methods

2.1. General

2.1.1. Chemicals

Folic acid (PteGlu) was provided by Sigma Aldrich (solid I, purity $\geq 97\%$) and Schircks Laboratories (solid II, purity $> 98.5\%$). Other pterins derivatives were purchased from Schircks Laboratories. Ammonium acetate (NH_4Ac) and formic acid (HCOOH) were purchased from Sigma Chemical Co. Acetonitrile (ACN) was purchased from J. T. Baker. Aqueous solutions were prepared using ultrapure water from Milli-Q® purification system (Millipore Corporation, USA).

2.1.2. Measurements of pH

A pH-meter sensION+ pH31 GLP combined with a pH electrode 5010T (Hach) or microelectrode XC161 (Radiometer Analytical) were used. The pH of the aqueous solutions was adjusted by adding drops of 0.1–0.2 M aqueous NaOH or HCl solutions with a micropipette. The concentration of the acid and the base used for this purpose ranged from 0.1 M to 2 M.

2.1.3. UV/vis Spectrophotometric Analysis

Electronic absorption spectra were recorded at room temperature on a Shimadzu UV-1800. Measurements were made using quartz cells of 0.4 and 1.0 cm optical path length.

2.2. Steady-state Irradiation

2.2.1. Irradiation Setup

Air equilibrated aqueous solutions containing PteGlu were irradiated in quartz cells (1.0 cm optical path length) at room temperature using a Rayonet RPR lamp (Southern N. E. Ultraviolet Co.) with emission centered at 350 nm (bandwidth ~ 20 nm).

2.2.2. Actinometry

Aberchrome 540 (Aberchromics Ltd.) was used as an actinometer for the measurement of the incident photon flux density ($q_{n,p}^{0,V}$) at the excitation wavelength, which is the amount of incident photons per time interval ($q_{n,p}^0$) and divided by the volume (V) of the sample [30]. Aberchrome 540 is the anhydride form of the (*E*)- α -(2,5-dimethyl-3-furylethylidene)(isopropylidene)succinic acid which, under irradiation in the spectral range 316–366 nm leads to a cyclized form. The method for the determination of $q_{n,p}^{0,V}$ has been described in detail [31].

The value of $q_{n,p}^{0,V}$ was $3.7 (\pm 0.4) \times 10^{-6}$ Einstein $\text{L}^{-1} \text{s}^{-1}$. The value of the absorbed photon flux density ($q_{n,p}^{a,V}$) was calculated from $q_{n,p}^{0,V}$ according to the Lambert-Beer law:

$$q_{n,p}^{a,V} = q_{n,p}^{0,V} (1 - 10^{-A}) \quad (1)$$

where A is the absorbance of the reactant at the excitation wavelength.

2.3. High-performance Liquid Chromatography (HPLC)

A Prominence equipment from Shimadzu (solvent delivery module LC-20AT, on-line degasser DGU-20A5, communications bus module CBM-20, auto sampler SIL-20A HT, column oven CTO-10AS VP, photodiode array (PDA) detector SPD-M20A and fluorescence (FL) detector RF-20A) was employed for monitoring the photochemical processes and to purify the PteGlu commercial sample. A Synergi Polar-RP column (ether-linked phenyl phase with polar endcapping, 150×4.6 mm, $4 \mu\text{m}$, Phenomenex) was used for isolation of PteGlu from HPLC runs (preparative HPLC). The column temperature was set at 25°C and the flow rate at 0.6 mL/min. Solution containing 100% NH_4Ac (1 mM, $\text{pH} = 6.7 \pm 0.1$) was used as mobile phase. The same column and runs conditions were used to analyze the purity of the isolated PteGlu sample and the progress of the photochemical processes.

2.4. Fluorescence Spectroscopy

Steady-state and time-resolved fluorescence measurements were performed on air-equilibrated aqueous solutions using a Single-Photon-Counting equipment FL3 TCSPC-SP (Horiba Jobin Yvon). The equipment has been previously described in detail [32].

In steady-state measurements the sample solution in a quartz cell was irradiated with a 450 W Xenon source through an excitation monochromator (FL-1004). The fluorescence, after passing through an emission monochromator (iHR320), was registered at 90° with respect to the incident beam using a room-temperature R928P detector. The emission measurements were performed at 25°C . Corrected fluorescence spectra obtained by excitation at 340 nm were recorded in the range 375–580 nm. The total fluorescence intensities (I_F) were calculated by integration of the fluorescence band centered at ca. 440 nm.

The fluorescence quantum yields (Φ_F) were determined from the corrected fluorescence spectra using the following equation:

$$\Phi_F = \Phi_F^R \frac{I_A^R}{I^R A} \quad (2)$$

where I is the integrated intensity, A is the absorbance at the excitation wavelength and the superscript R refers to the reference fluorophore. In our experiments, pterin in acid medium ($\text{pH} = 6.0$) was used as a reference ($\Phi_F = 0.32$) [33]. The sample and reference were excited at the same wavelength. To avoid inner filter effects, the absorbance of the solutions at the excitation wavelength was kept below 0.10.

In time-resolved experiments a NanoLED source (maximum at 341 nm) was used for excitation and the emitted photons, after passing through a monochromator, were detected by a TBX-04 detector and counted by a FluoroHub-B module.

2.5. Mass Spectrometry Analysis

The liquid chromatography mass spectrometry system was equipped with an UPLC chromatograph (ACQUITY UPLC from Waters) coupled to a quadrupole time-of-flight mass spectrometer (Xevo G2-QToF-MS from Waters) (UPLC-QToF-MS). UPLC analyses were performed using the Acquity UPLC BEH Phenyl ($1.7 \mu\text{m}$, 2.1×50 mm) column (Waters). An isocratic elution with 90% of aqueous HCOOH (0.1% v/v) and 10% of ACN was used as mobile phase with a flow rate of 0.2 mL/min. Mass chromatograms, *i.e.* representations of mass spectrometry data as chromatograms (the x-axis representing time and the y-axis signal intensity), were registered using different scan ranges. The mass spectrometer was operated in both positive (ESI^+) and negative (ESI^-) ion modes.

Table 1Values of m/z recorded for the molecular ions in MS spectra of the compounds present in a solution of PteGlu (100 μM) prepared using solid I.

Compound	Elemental composition [M]	ESI ⁺ [M + H] ⁺			ESI [−] [M − H] [−]		
		Observed m/z	Calculated m/z	Error (ppm)	Observed m/z	Calculated m/z	Error (ppm)
PteGlu	C ₁₉ H ₁₉ N ₇ O ₆	442.1472	442.1470	0.45	440.1324	440.1324	0.00
PABA-Glu	C ₁₂ H ₁₄ N ₂ O ₅	267.0981	267.0975	2.25	265.0823	265.0830	−2.64
Fop	C ₇ H ₅ N ₅ O ₂	192.0529	192.0516	6.77	190.0367	190.0370	−1.58
Cap	C ₇ H ₅ N ₅ O ₃	208.0478	208.0465	6.25	206.0323	206.0320	1.46

3. Results and Discussion

3.1. Identification of the Impurities Present in Commercial PteGlu

Most of the PteGlu commercial solids used for photochemical studies are of suitable purity and are used without further purification. Nevertheless little is known about the chemical nature of the impurities. To the best of our knowledge, only one work has investigated this issue and Cap and PABA-Glu have been reported as impurities of commercial PteGlu [34].

In order to further investigate the impurities, aqueous solutions of PteGlu were prepared ($\text{pH} = 6.0 \pm 0.1$) from commercial solids of different manufacturers (Materials and Methods section) in the absence of light and the samples were analyzed by liquid chromatography/mass spectrometry (LC/MS), using a UPLC equipment coupled to a mass spectrometer (UPLC-QToF-MS, Materials and Methods section). The chromatograms of solutions recorded using the UV/vis detector, showed only one peak at retention times (t_r) of 2.10 min (Fig. 2S, Supplementary material). Analyses were performed in both positive and negative ion modes (ESI⁺ and ESI[−], respectively). For the peak at t_r 2.10 min, the signals corresponding to the intact molecular ion of PteGlu as $[\text{M} + \text{H}]^+$ and $[\text{M} - \text{H}]^-$ species were observed ($\text{M} = \text{PteGlu}$) (Table 1). The mass spectra recorded in both ESI⁺ and ESI[−] modes for different t_r values revealed the presence of several compounds (Figs. 3S and 4S, Supplementary material). As shown in Table 1, three of the ions detected in the mass spectra clearly correspond to Fop, Cap and PABA-Glu.

The samples were then analyzed by HPLC coupled to a PDA-detector (Materials and Methods section). 75 μL of a solution of PteGlu (20 μM) prepared from solid I were injected and chromatograms at different analysis wavelengths were recorded, all of them showing only one peak at t_r 4.9 min (Fig. 1a). The spectrum registered for this peak corresponded to the spectrum of PteGlu solutions recorded in a spectrophotometer. This first analysis suggested a suitable purity of the sample. However, when more concentrated solutions were injected new peaks with low intensity appeared (inset Fig. 1a), revealing the presence of several impurities at low concentrations. Absorption spectra were recorded for these impurities and two of them at t_r values of 1.9 and 12.5 min were compatible with spectra of PABA-Glu and Fop, respectively (inset Fig. 1a).

The solutions were also analyzed by HPLC using the FL-detector (Materials and Methods section). The conditions for the analysis were chosen taking into account the fluorescence properties of unconjugated pterins [33]: excitation wavelength (λ_{exc}) = 350 nm, emission wavelength (λ_{em}) = 450 nm. The chromatograms registered under these conditions showed 4 and 2 peaks for the solutions prepared with solid I and II, respectively (Fig. 1). The comparison of the t_r values observed in these chromatograms with those obtained with a series of solutions of standards of unconjugated pterins revealed that two of the impurities are Cap and Fop. In another set of analyses, taking into account the fluorescent properties of PABA-Glu [35], the optimal conditions for the detection of this compound were set: λ_{exc} = 270 nm, λ_{em} = 350 nm. The chromatograms registered under these conditions (inset Fig. 1b) showed a peak with the same t_r value as that observed for a standard solution of PABA-Glu.

In addition, the percentage of each impurity was determined in both commercial solids. For this purpose, equal masses of solid I and II, were dissolved in the same volume of H₂O ($\text{pH} = 6.0 \pm 0.1$) in dark conditions. After that, the same volume of each solution was analyzed by HPLC. The amount of each impurity was determined by integration of the peaks of the chromatograms recorded with the FL-detector, using the calibration curves of each compound. The values obtained are presented in Table 2.

Results presented in this section showed that usual commercial PteGlu is unpurified with unconjugated oxidized pterins. These compounds are in such low amounts that they are not detected by HPLC using spectrophotometric detection, which is the most usual in chromatography. In consequence a normal chromatographic control of commercial PteGlu would not detect any pterinic contamination.

3.2. Purification of PteGlu

To assess the effect of the oxidized pterins, present as impurities, on the spectroscopic and photochemical properties of the PteGlu aqueous

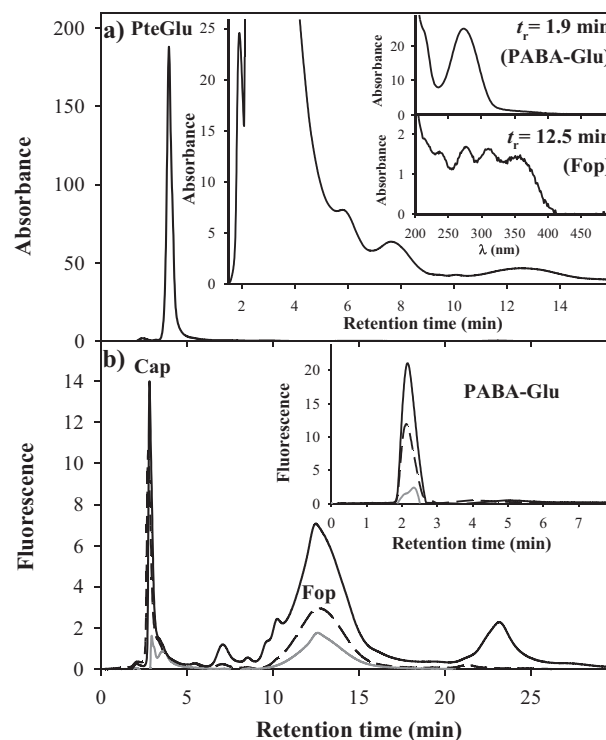


Fig. 1. HPLC analysis of aqueous solution of PteGlu. a) Chromatograms of solutions prepared from solid I using the PDA detector at 270 nm. Main graph: chromatogram recorded after injecting 75 μL of a solution 20 μM ; inset: chromatogram recorded after injecting 75 μL of a solution 240 μM and absorption spectra of the peaks with t_r 1.9 and 12.5 min. b) Chromatograms obtained using the FL detector of solutions prepared from solid I (solid black line), solid II (dashed line) and PteGlu purified (gray line); λ_{exc} = 350 nm, λ_{em} = 450 nm; inset: λ_{exc} = 270 nm, λ_{em} = 350 nm. [PteGlu] = 20 μM , $\text{pH} = 6.0$.

Table 2

Amount of each impurity (expressed in mol) per 100 mol of PteGlu in solutions prepared from different commercial solids (Materials and Methods section) and with purified PteGlu.

PteGlu solution	PABA-Glu	Cap	Fop
Solid I	1.0 ± 0.2	0.17 ± 0.01	1.3 ± 0.1
Solid II	0.7 ± 0.2	0.16 ± 0.02	0.7 ± 0.2
Purified PteGlu	0.3 ± 0.1	0.014 ± 0.006	0.2 ± 0.1

solutions prepared from commercial solids, PteGlu was isolated from HPLC runs (Materials and Methods section). We decided to purify the PteGlu present in solutions prepared from solid II since it is the purest one. PteGlu was collected using NH₄Ac aqueous solution (1 mM, pH = 6.7 ± 0.1) as an eluent, the pH of the resulting solution was adjusted at 6.0 ± 0.1 and the absorption spectrum was controlled.

The HPLC analysis of the collected fraction suggested that the isolation of PteGlu reduced significantly the amount of each impurity present in the sample (Fig. 1, Table 2), but they could not be totally eliminated. It is noteworthy that while the relative concentration of Cap decreased to less than 10% of the initial one, the elimination of Fop upon purification was much less efficient (Table 2).

3.3. Fluorescence Properties

The fluorescence properties of the solutions of PteGlu purified and unpurified were studied and compared. Corrected fluorescence spectra of the three solutions obtained by excitation at 340 nm were recorded in the range of 375–580 nm, under the same experimental conditions (absorbance at the excitation wavelength (λ_{exc}), pH 6.0, slits of the excitation and emission monochromators, etc.). As shown in Fig. 2, the more unpurified is the sample, the more intense is the emission spectra. Moreover, the apparent fluorescence quantum yields (Φ_F) were calculated using Ptr in aqueous solution (pH 5.5) as a reference ($\Phi_F = 0.32 \pm 0.01$) [33], and the value of this parameter decreased with the concentration of Fop and Cap in the sample (Table 3). These results indicate that the fluorescent emission of a solution of PteGlu is governed by the unconjugated pterins present as impurities and not by the PteGlu itself.

To quantify the contribution of Fop and Cap to the total

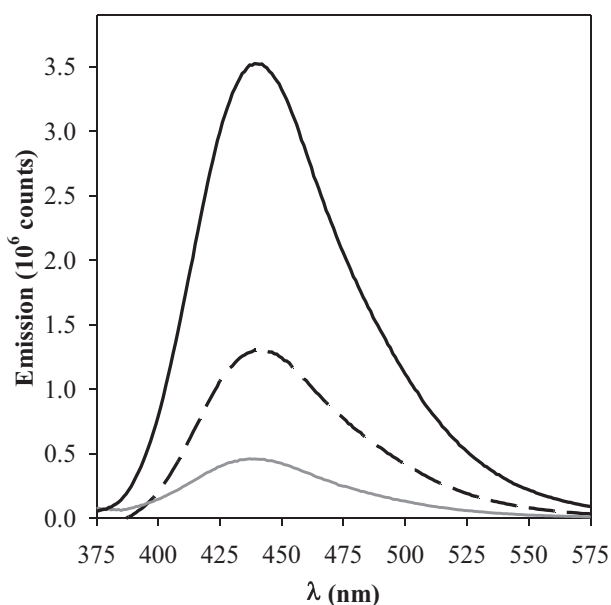


Fig. 2. Corrected fluorescence spectra ($\lambda_{\text{exc}} = 340$ nm) of an aqueous solution of purified (gray solid line) and unpurified folic acid obtained from solid I (black solid line) and solid II (black dashed line); the absorbance at λ_{exc} was 0.1 for all the samples, pH = 6.0 ± 0.1.

fluorescence of the solutions of PteGlu, the peaks of the chromatograms recorded using the FL-detector (Fig. 1) were integrated. The percentage of the area of each peak corresponds to the percentage of the fluorescence of the solution emitted by each compound under the conditions chosen for the detection ($\lambda_{\text{exc}} = 350$ nm, $\lambda_{\text{em}} = 450$ nm). As shown in Table 3, even in the solution of purified PteGlu, the compounds responsible for the fluorescence of the solution upon excitation at 350 nm are Fop and Cap. It is worth mentioning that in the case of the solutions prepared from solid I, the HPLC analysis showed the presence of other fluorescent compounds different from Fop and Cap that also contribute to the emission of the solution upon excitation with UV-A radiation.

In time-resolved experiments, fluorescence decays were analyzed for the different solutions studied. The solutions prepared from solid II and the purified PteGlu were fitted with three exponential functions and the obtained lifetimes values (τ_F) with their corresponding relative intensities are shown in Table 3. For both samples the two most intense components presented τ_F values (τ_{F2} and τ_{F3}) very close to those previously reported for Cap and Fop, respectively ($\tau_{F(\text{Fop})} = 7.9 (\pm 0.4)$ ns, $\tau_{F(\text{Cap})} = 5.8 (\pm 0.4)$ ns) [33]. In addition, the relative fluorescence intensity of the two components is quite similar to the relative emission of Fop and Cap calculated in the corresponding solutions from chromatograms recorded using the FL-detector (Table 3). Finally, it is worth mentioning that kinetic analysis of the decays recorded for solutions prepared from solid I could not be carried out fitting with up to four exponential functions, which is not surprising due to the considerable presence of other fluorescent impurities in addition to Fop and Cap (Table 3).

Therefore, the time-resolved study performed with solutions prepared from solid II and the purified PteGlu, in agreement with fluorescence steady-state experiments and HPLC-FL analysis, indicate that fluorescence of Fop dominates the emission of the analyzed samples and Cap also contributes. The third fluorescent detected component (τ_{F1}) might correspond to PteGlu, but more studies should be done to determine correctly its τ_F value.

The results presented in this section showed that the fluorescence of a solution of PteGlu prepared using commercially available solids cannot be attributed to PteGlu and the fluorescence of Fop and Cap present as impurities is what really is measured. This fact occurs because the real fluorescence of PteGlu is extremely weak. The Φ_F value of PteGlu is negligible compared to those of Fop and Cap, therefore although these compounds are in very small concentrations in the solution, their fluorescence dominates the emission of the PteGlu solution. These findings suggest that many works reporting data about the fluorescence of PteGlu should be revised.

3.4. Stability of PteGlu Under UV-A Radiation

Many reports have studied the photodegradation of PteGlu in aqueous solutions under UV-A radiation (see Introduction). Nevertheless, no one has investigated the effect of the pterinic impurities on the photostability of PteGlu. Therefore, air equilibrated aqueous solutions prepared from solids I and II and purified PteGlu, were exposed to UV-A irradiation for different periods of time. The irradiated samples were analyzed by HPLC and the concentration profiles of PteGlu, Fop and Cap were obtained.

The initial rate of PteGlu consumption ($-(d[\text{PteGlu}]/dt)_0$) was calculated from the corresponding plots of PteGlu concentration as a function of irradiation time (Fig. 3a) and values of 1.1 (± 0.1) $\mu\text{M min}^{-1}$, 0.41 (± 0.04) $\mu\text{M min}^{-1}$ and 0.046 (± 0.008) $\mu\text{M min}^{-1}$ were obtained for solutions prepared from solids I and II and purified PteGlu, respectively. It is clear that $-(d[\text{PteGlu}]/dt)_0$ increased with the concentration of pterinic impurities, thus indicating that initial consumption of PteGlu depends on the presence of Fop and Cap in the medium. A similar behavior was observed for the formation of Fop (Fig. 3b), that is, the higher the purity of the solution, the lower the rate of Fop formation. These results suggest that PteGlu is not degraded by

Table 3

Apparent fluorescence quantum yields (Φ_F) and fluorescence lifetime (τ_F) calculated for solutions prepared from solid I, solid II and purified PteGlu ($\lambda_{exc} = 350$ nm, pH = 6.0); Relative Emission calculated from chromatograms recorded using the FL-detector (Fig. 1, $\lambda_{exc} = 350$ nm, $\lambda_{em} = 450$ nm).

PteGlu solution	Φ_F (10^{-4})	τ_{F1} (ns)	τ_{F2} (ns)	τ_{F3} (ns)	Relative emission (%)			
					PteGlu	Cap	Fop	Others
Solid I	51 ± 5	–	–	–	2.3 ± 0.6	11 ± 2	67 ± 8	20 ± 6
Solid II	21 ± 2	0.5 (5%)	4.3 (20%)	8.8 (75%)	4 ± 1	22 ± 5	67 ± 7	8 ± 2
Purified PteGlu	7 ± 1	0.5 (10%)	3.9 (20%)	8.6 (70%)	12.1 ± 0.7	16 ± 3	73 ± 7	–

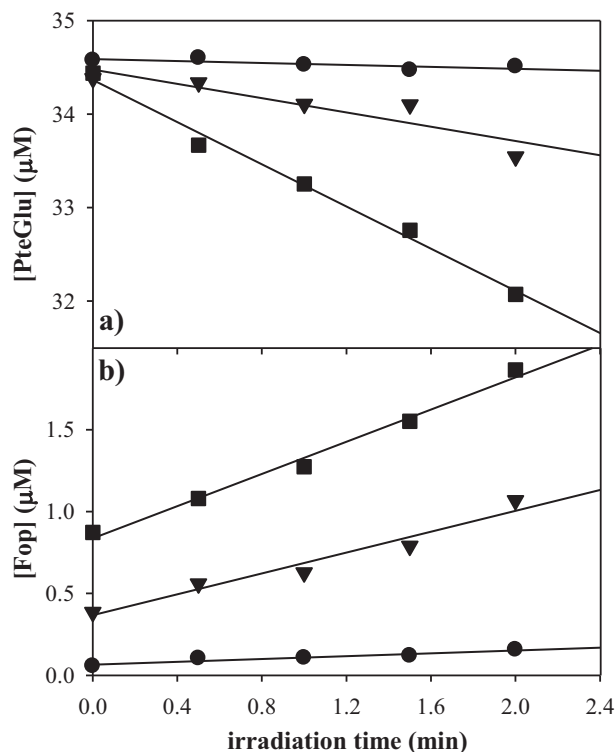


Fig. 3. Time-evolution of a) PteGlu and b) Fop concentrations during irradiation of air-equilibrated aqueous solution of purified PteGlu (●), unpurified PteGlu obtained from commercial solid I (■) and II (▼). pH = 6.0 ± 0.1 .

direct excitation of its pterinic moiety, but by photosensitization with unconjugated pterins present in the solutions as impurities. A similar behavior was also observed in other pterin derivatives, such as dihydro and tetrahydropterins. In this case, the reduced pterins suffer auto-oxidation yielding aromatic pterins as products, which are able to sensitize the photodegradation of reduced pterin [36].

The behavior observed for longer irradiation times (Fig. 4) was in agreement with previous studies, that is, after a period of time the rate of PteGlu degradation increased with time [22,24] due to the accumulation of products (oxidized unconjugated pterins) that are able to photosensitize PteGlu. Although this behavior was observed for the three samples analyzed, the acceleration in the consumption of PteGlu occurred at different irradiation times: the more unpurified the sample, the shorter the time for the acceleration.

Therefore the rate of the overall photodegradation of PteGlu upon UV-A radiation depends on the initial concentration of oxidized pterins present in the solution. The results shown in this section suggested that when the photolysis of PteGlu is studied using aqueous solutions prepared from commercially available solids, as a matter of fact, the photodegradation of this compound initiated by the direct absorption of photons is not investigated. Instead of that, what is really analyzed is the degradation of PteGlu photosensitized by oxidized unconjugated pterins.

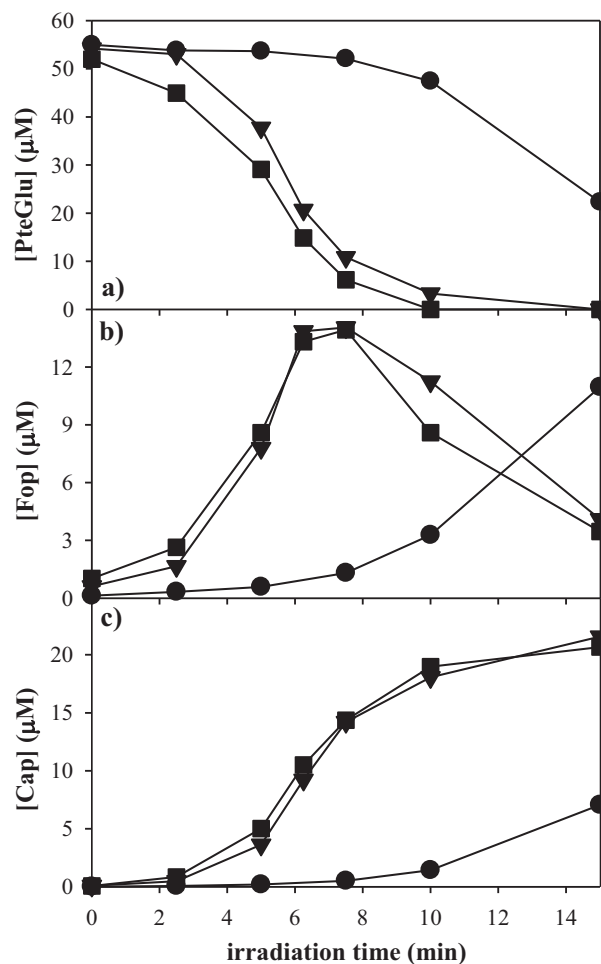


Fig. 4. Time-evolution of PteGlu, Fop and Cap concentrations during irradiation of an air-equilibrated aqueous solution of purified PteGlu (●), unpurified PteGlu obtained from commercial solid I (■) and II (▼). pH = 6.0 ± 0.1 .

4. Conclusions

Usual commercial PteGlu is unpurified with unconjugated oxidized pterins, in particular, 6-formylpterin (Fop) and 6-carboxypterin (Cap). These compounds are in such low amounts that they are not detected by HPLC using spectrophotometric detection, which is the most usual in chromatography to control purity. PteGlu was isolated from HPLC runs and the concentration of the impurities was reduced significantly. The fluorescence and photochemical properties of the PteGlu aqueous solutions prepared from commercial solids and the purified solutions were compared to assess the effect of the oxidized pterins, present as impurities.

The fluorescence of PteGlu solutions is due to the emission of Fop and Cap and the contribution of the PteGlu emission is negligible. This is because the fluorescence quantum yield (Φ_F) of PteGlu is extremely weak compared to the Φ_F of Fop and Cap. Likewise, the PteGlu

photodegradation upon UV-A radiation is an oxidation reaction photosensitized by oxidized unconjugated pterins present in the solution, and not a process initiated by the direct absorption of photons by PteGlu.

The results presented have important implications because the fluorescence and photochemical properties of PteGlu solutions, prepared using commercially available solids, are dominated by the unconjugated pterins present as impurities and not by PteGlu itself. This fact calls into question many reported studies on fluorescence and photooxidation of this compound. Moreover, the photodegradation of PteGlu in biological systems might be consequence of photosensitized processes and the direct excitation of PteGlu might play a minor role.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jphotobiol.2018.03.007>.

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