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Mechanism of folic acid radiolysis in aqueous solution

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ABSTRACT

Folates compounds are a B group vitamin vital for important biochemical processes like DNA synthesis and repair and in certain biological reactions as a cofactor. Folic acid (FA) is composed of a pteridine ring, *p*-aminobenzoic acid and glutamate moieties. Separately, the three moieties have no vitamin activity. Folate deficiency can lead to increased risk of several pathologies. FA is known to be a sensitive compound, easily degraded by pH, light, heat and food processing. Food irradiation is a process exposing food to ionizing radiations to reduce storage losses, extend shelf life and microbiological safety. Radiation treatment produces oxygen radicals and thereby induces oxidative damage in biomolecules such as proteins, lipids, DNA and vitamins. In the present work, aqueous FA solutions are submitted to electron-beam (E-beam) radiation in a dose range of 0.25–10 kGy. Upon irradiation, main FA radio-products are quantified by HPLC. E-beam processing undergoes radiolysis to yield some known FA photoproducts and also new radio-products are formed: 6-(hydroxymethyl)pterin and N-(4-nitrobenzoyl)-L-glutamic acid. A radio-degradation pathway of FA is also discussed.

1. Introduction

Folate compounds are a B group vitamin, vital for important biochemical processes like DNA synthesis and repair and in certain biological reactions as a cofactor (Lucock, 2000).

Folate deficiency can induce diseases such as megaloblastic anemia, neural tube defects, cardiovascular diseases, cancer and has also been associated with age-related cognitive decline, particularly in older adults (Durga et al., 2007; Moat et al., 2004; Rucker, Suttie, McCormick, & Machlin, 2001). FA is the synthetic form of folate and is found in food supplements and fortified food. FA is used for this purpose because it is cheaper and more stable than physiological types of folate (McNulty & Pentieva, 2004). Folic acid (FA) or pteroylglutamic acid is composed of a pteridine ring, *p*-aminobenzoic acid (PABA) and glutamate moieties. Separately, the three moieties have no vitamin activity (Lucock, 2000; USP, 1975).

Food treatments intend the preservation of nutritional quality and microbiological safety during food processing. The best compromise between the goal of the food treatment and the

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preservation of nutritional quality involves optimizing the intensity of treatment and knowledge of the transformation process. Radiation treatment is a process exposing food to ionizing radiations such as gamma rays emitted from the radioisotopes ^{60}Co or high energy electrons and X-rays produced by machine sources (Farkas, 2006). Irradiation produces oxygen radicals and thereby induces oxidative damage in biomolecules such as proteins, lipids and DNA (Riley, 1994). FA is known to be a sensitive compound, easily degraded by pH, light, heat and food processing (Ball, 2006; Delchier et al., 2014; Hefni & Witthöft, 2014).

In recent years, the agrifood sector, due to the globalization and the development of new technologies, is undergoing radical changes that require a deeper characterization of the food chain, starting from raw materials up to the final products. In addition, the consumers are concerned about the food they eat and ask for more assurances on the quality, the safety and the geographical origin of the products that they consume (Di Stefano et al., 2012). Despite few reports on FA radio-degradation found in literature (Araújo et al., 2011; El-Dessouky, Abd-Elwahab, & Turk, 1988; Kesavan, Pote, Batra, Viswanathan, 2003; Nakken & Pihl, 1966) much is still let to be known, specially on the mechanism of FA radiolysis. The purpose of the present work is to identify and quantify by HPLC the radio-degradation products of folic acid aqueous solutions submitted to increasing doses of ionizing radiation and to investigate the possible radio-degradation mechanism of this vitamin.

2. Materials and methods

2.1. Chemicals

Methanol (MeOH) (Carlo Erba, Val de Reuil, France) were HPLC grade. All other chemicals were of analytical grade. Acetic acid was purchased from Riedel-de Haen (Seelze, Germany). Sodium hydroxide (NaOH) was purchased from SDS (Peypin, France). FA was obtained from Sigma–Aldrich (Steinheim, Germany). FA degradation products, xanthopterin monohydrate (XA) was purchased from Acros Organics (Geel, Belgium), 6-(hydroxymethyl)pterin (AHMP) was obtained from Florida Center for Heterocyclic Compounds (Florida, USA) and pterine-6-carboxylic acid (PCA), *p*-aminobenzoyl-L-glutamic acid (pABGA), 4-aminobenzoic acid (PABA), N-(4-nitrobenzoyl)-L-glutamic acid hemihydrate (pNBGA), pteroic acid (PA) were obtained from Sigma–Aldrich (Steinheim, Germany). Water was purified using a Synergy Milli-Q System (Millipore, Molsheim, France).

2.2. Samples

All FA aqueous solutions were prepared under subdued light, to protect folates from oxidative degradation induced by light. A total of 100 mg of FA was added to 100 mL of water and solubilized by the addition of 5.0 mL of 1 mol/L NaOH. The solution was diluted in water to a final concentration of 100 μg/mL (pH 8.3). All samples (4 mm thick) were packed in polyamide/polyethylene plastic sachets, sealed and labeled with their respective radiation doses. PABA, pABGA and pNBGA were dissolved in water. PCA, PA, XA and AHMP were dissolved in water with addition of NaOH 1 mol/L until solubilization. All degradation products standards solutions were diluted in water at a final concentration of 100 μg/mL.

2.3. Irradiation treatment

FA aqueous solutions were irradiated in five replicates in a Van de Graaff E-beam accelerator, 2 MeV (Vivirad High Voltage, Handschuheim, France) with a 100 μA current, 20 cm scan width, and about 2 kGy/s dose rate. Applied doses were around 0.25, 0.50, 0.75, 1.0, 3.0, 5.0, 7.0, 10.0 kGy and 0 (control). Surface absorbed doses were monitored with FWT 60.00 radiochromic dosimeters (Far West Technology, Goleta, CA), previously calibrated with an alanine dosimeter (Aérial, Illkirch, France) (Kuntz, Pabst, Delpech, Wagner, & Marchioni, 1996). Dose uniformity of about 10% within the sample was achieved by the use of a 100 μm thick copper scattering foil (Kuntz, Marchioni, & Strasser, 1991). Standard conditions for temperature and pressure were used in irradiation plant (25 °C and 1 atm).

2.4. HPLC-MS

Experimental work was performed using a Varian ProStar HPLC system (Palo Alto, CA) [210 binary HPLC pump, 410 AutoSampler and 335 diode array detection (DAD) UV–Vis detector] and a Varian 1200L triple quadrupole mass spectrometer system (MS) (Varian, LesUlis, France). LC/UV and LC/MS were used respectively for quantification and identification purposes. High-purity nitrogen (Domnik Hunter, Villefranche sur Saône, France) was used as nebulizing gas (344.73 kPa) and drying gas (200 °C). Needle and shield voltage were 5000 V and 600 V, respectively. The spectrometer was operated in full scan mode (70–500 m/z) using positive electrospray ionization (ESI+). Chromatographic separation was carried out on an Agilent-XDB Phenyl analytical column (250 mm × 4.6 mm; 5 μm particle size) and the eluate was split 1:5 to MS detector and 4:5 to DAD detector. Mobile phase consisted of aqueous acetic acid (0.5%) and MeOH, at a flow rate of 1.0 mL/min. Gradient elution started at 100% aqueous acetic acid for 10 min, followed by raising the MeOH concentration linearly to 45% within 42 min. Subsequently, MeOH concentration decreased to 0% in 1 min and then the column was equilibrated at this condition for 12 min. The injection volume was 20 μL. Detection was performed at 280 nm. A negative (H_2O) and a positive control (100 μg/mL FA aqueous solution) were injected throughout the sample analysis every 10 injections to control the analytical system.

2.5. Quantification of FA and FA degradation products

Prior to quantification, FA aqueous solutions (irradiated and non-irradiated) were pooled together and concentrated 10 times at 40 °C using a SpeedVac concentrator Savant SPD 121P (Thermoscientific, EUA) in order to allow the quantification of the less generated radio-products. Quantification of FA, XA, pABGA, pNBGA, AHMP and PCA was performed by external calibration by plotting peak areas versus compounds concentrations. Linear calibration curves were obtained from 1.6 to 100.0 μg/mL using at least six calibrators for each compound. Limit of detection (LOD) and limit of quantification (LOQ) values determination were based on the standard deviation of residuals according to ICH Q2B (1996). Briefly, they were calculated as the ratio between the standard deviation of residuals and the slope of calibration curve. The obtained value was multiplied by 3.3 or 10, respectively to LOD and LOQ.

3. Results and discussion

E-beam processing degraded intensively FA molecule (Fig. 1A–D). Degradation of FA molecule was followed by the formation of several degradation products (Fig. 1). Increasing radiation doses enhanced FA breakdown up to 10 kGy. It can be assumed that with radiation doses up to 1 kGy, mainly products with low-polarity were formed, nearby FA retention time (42.4 min). Radiation doses over 1 kGy degraded intensely FA molecule into more polar fragments. These results are in accordance with others

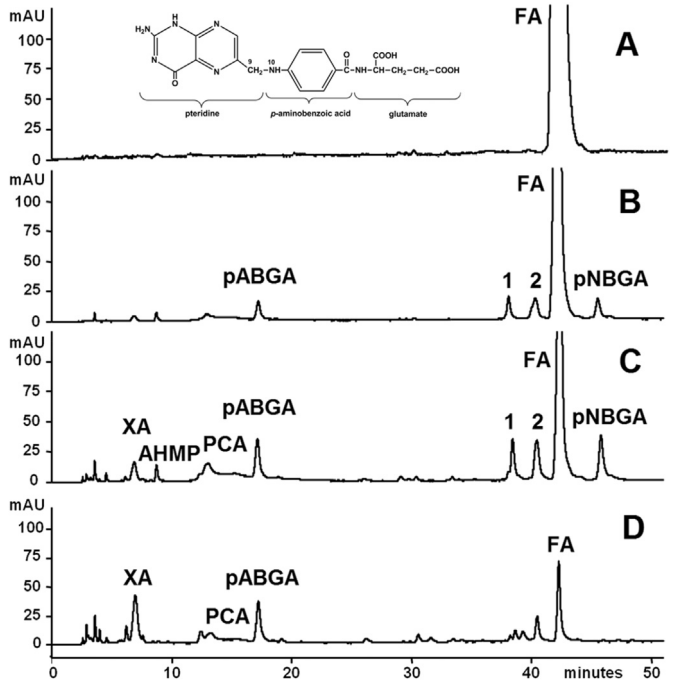

Fig. 1. LC/UV profiles of FA solutions after E-beam irradiation with 0 kGy (A), 0.25 kGy (B), 1.0 kGy (C) and 5.0 kGy (D). Inset: FA (folic acid) molecule. XA (xanthopterin); AHMP (6-(hydroxymethyl)pterin); PCA (pterine-6-carboxylic acid); pABGA (p-aminobenzoyl-L-glutamic acid); Peak 1 (unknown); Peak 2 (unkown); pNBGA (N-(4-nitrobenzoyl)-L-glutamic acid hemihydrate).

studies on FA radio-stability (Araújo et al., 2011; Araújo et al., 2012; El-Dessouky et al., 1988; Kesavan et al., 2003). During the development of the proposed LC/UV method, gradient conditions were optimized including an increase of organic phase (MeOH) concentration up to 100%. Thus, it was observed that no more detectable peaks eluted in the chromatographic run after 46 min in FA irradiated solutions.

According to their increasing retention times, the main radio-products of FA were identified as: xanthopterin (XA), 6-(hydroxymethyl)pterin (AHMP), pterine-6-carboxylic acid (PCA), *p*-aminobenzoyl-L-glutamic acid (pABGA) and N-(4-nitrobenzoyl)-L-glutamic acid (pNBGA) (Fig. 1). Identification of these FA radio-products performed by LC-MS/MS analysis is in agreement with previous studies on FA degradation (Araújo et al., 2012; El-Dessouky et al., 1988; Kesavan et al., 2003). Stability of folates and FA has been already studied by several authors, notably the effect of photolysis (Akhtar, Khan, & Ahmad, 2003; Dántola et al., 2010; Off et al., 2005; Thomas, Suarez, Cabrerizo, Martino, & Capparelli, 2000; Vorobey, Steindal, Off, Vorobey, & Moan, 2006) but also some studies on radio (El-Dessouky et al., 1988; Müller & Diehl, 1966; Nakken & Pihl, 1966) and thermal stability (Vora, Riga, Dollimore, & Alexander, 2002). In general, these studies stated that FA could degrade into different degradation products depending on cleaving sites of FA molecule, including mainly pterine-6-carboxylic acid (PCA), *p*-aminobenzoyl-L-glutamic acid (pABGA), *p*-aminobenzoic acid (PABA), pteroic acid (PA) and glutamic acid (GLU). In our study PABA traces were found in LC/ESI + chromatograms (spectrum not shown) of FA solutions treated with radiation doses close to 1 kGy. Regarding to GLU, this very polar molecule exhibited a poor retention with the applied reversed-phase chromatographic conditions, eluting near the solvent front. No important increase in solvent peak was observed, thus it was assumed that GLU presence was not relevant in irradiated samples.

The presence of pABGA and PCA in the irradiated FA solutions indicated a cleavage in C_9—N_{10} bond of FA molecule (Fig. 1 inset). Previous studies on FA photolysis have already proposed pABGA and PCA as photoproducts (Akhtar, Khan, & Ahmad, 1997; Off et al., 2005; Suárez, Cabrerizo, Lorente, Thomas, & Capparelli, 2000; Thomas et al., 2002; Thomas et al., 2000). These authors concluded that FA first undergone a cleavage at C_9—N_{10} bond with pABGA and 6-formylpterin (FPT) formation. Further photo-radiation leaded to photo-oxidation of the 6-formyl group at FPT and formation of PCA (Vorobey et al., 2006). Our findings supported partially this hypothesis since contrarily to PCA, FPT was not found in irradiated FA solutions. This evidence could be explained due to the high energy involved during E-beam processing which could accelerate PCA formation. It is noteworthy that E-beam treatment is an in-deep treatment, more energetic and with higher dose rates compared to photolysis. That is why E-beam treatment promoted an intense FA radiolysis. A few studies were done on FA radio-degradation. Nakken and Pihl (1966) evaluated the X-ray induced damage of FA, PABA, pABGA and N-(*p*-aminobenzoyl)glycine in phosphate solutions (pH 7.4). They proposed that radiation-induced hydroxyl radicals cleaved C_9—N_{10} bond of FA molecule. Moreover, these authors found two other molecules, 3-OH-pABGA and *o*-aminophenol.

In a previous work, Araújo et al. (2012) studied the effect of E-beam treatment in FA aqueous solutions. A non-conjugated pteridine compound was identified as a new radio-product, named xanthopterin (XA). In the present work, two new FA radio-products were identified by LC-MS/MS and confirmed towards their standards. The first identified compound exhibited in its MS spectra a m/z value of 297.0 $[M+H]^+$. Its identity was confirmed towards a standard of N-(4-nitrobenzoyl)-L glutamic acid (pNBGA). This new radio-product shares a similar molecular structure to pABGA, differing from the latter due to the substitution of NH_2 functional group to a NO_2. The second new radio-product showed in its MS spectra a m/z value of 194.0 $[M+H]^+$ which corresponded to a 6-(hydroxymethyl)pterin (AHMP) standard. Unlike to PCA, AHMP presents a substitution of the —COOH group to a CH_2OH in the non-conjugated pteridine structure. Identity of compounds eluting at 38.12 min (peak 1) and 40.37 min (peak 2) are still unknown and will be subject of future studies (Fig. 1).

The high energy involved during E-beam treatment also degraded FA into several less expressive radio-products (Fig. 1). Alike, a thermo-degradation study of FA revealed an intense FA breakdown related to increasing temperatures (Vora et al., 2002). These authors demonstrated by FTIR analysis (Fourier transform infrared spectroscopy) that as a function of temperature GLU was lost first, followed by the loss of the pterin. A complete loss of acid and amide functionality was realized by 195 °C, resulting in an amorphous product at 349 °C. Finally, FA was ultimately degraded to small carbon fragments. Ball (2006) also stated that FA has no well-defined melting point but darkens at 250 °C, followed by charring.

In order to reach a quantitative approach of FA degradation and radio-products formation, calibration curves were constructed by plotting peak areas versus concentrations of each degradation product standards. A linear response (at 280 nm) was found for FA and its degradation products over all the evaluated range (Table 1). Regression curve analyses resulted in excellent straight-line fits with correlation coefficients close to 1.000. FA and FA degradation products calibration data for the LC/UV method is summarized in Table 1.

Folic acid concentration and its degradation products were measured as function of the radiation dose applied (Fig. 2). Radiation doses as low as 0.75 kGy were enough to decrease by half the initial FA concentration (226 μmol/L). Concerning other FA

Table 1
Regression calibration data for the RP-HPLC method.

Analytes	Retention time (minutes)	Slope	Intercept[a]	Correlation (r^2)	LOD (ng)	LOQ (ng)
FA	42.4	109,120	413	0.999	74.0	224.3
XA	6.92	48,517	32	0.999	6.4	19.3
pABGA	17.29	419,782	805	0.999	22.8	69.1
pNBGA	45.78	44,921	46.7	0.999	30.5	92.4
AHMP	8.68	25,301	25.6	0.999	11.2	33.9
PCA	14.02	47,010	−153.2	0.999	15.7	47.6
PABA	16.49	47,580	34.5	0.999	3.2	9.8

[a] Intercept not significantly different from 0 at a significance level of 0.05.

radiolysis studies, El-Dessouky et al. (1988) studied the effect of gamma radiation on FA and its cobalt complex solutions. They observed that FA solutions (pH 9.2) absorptions gradually decreased with increasing absorbed doses from 2 to 10 krad as well as from 2 to 10 Mrad. Müller and Diehl (1996) analyzed the effect of ionizing radiation on folates in spinach, green cabbage and Brussels sprouts and revealed that a radiation dose of 2.5 kGy caused about 10% loss of total folates. It is important to highlight that the low water content of these samples and food matrix itself could explain this low FA percentage loss compared to our results with FA aqueous solutions. On the other hand, the effect of E-beam irradiation on hamburgers enriched with FA showed a 20–30% FA reduction following irradiation with a radiation dose of 2 kGy (Galán, García, & Selgas, 2010). Similarly, a study on the effect of E-beam treatment on the availability for intestinal absorption (bioaccessibility) of FA incorporated to ready to eat meat products reported a 20–30% loss of FA with 2 and 3 kGy doses (Galán, García, Selgas, & Havenaar, 2014). These authors also performed a reference experiment using FA enriched milk because it was the first time that a bioaccessibility test of FA fortified meat products were studied using a dynamic gastrointestinal model. A very strong negative effect of E-beam on the FA content of UHT milk was realized at a dose of 3 kGy, resulting in approximately 95% loss. These authors suggested that the presence of oxygen and water could profoundly influence the radiolytic process due to the accelerated electrons reacted with oxygen, giving radicals, which could affect to the FA oxidation. Our results are consistent with these findings, an 85% FA loss was found at a 3 kGy radiation dose (Fig. 2).

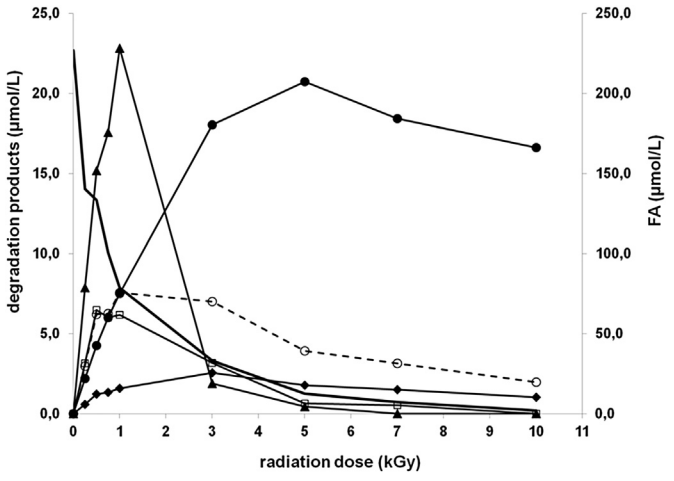

Fig. 2. Evolution of ▬ FA (folic acid) and FA radio-products concentrations in irradiated FA aqueous solutions as function of radiation dose (FA concentration on the right; FA degradation products concentration on the left). ●— XA (xanthopterin); □— AHMP (6-(hydroxymethyl)pterin); ◔— PCA (pterine-6-carboxylic acid); ◆— pABGA (*p*-aminobenzoyl-L-glutamic acid); ▲— pNBGA (N-(4-nitrobenzoyl)-L-glutamic acid hemihydrate).

Irrespective of applied radiation dose, the sum of individual concentrations of major FA radio-products (16.78–45.63 µmol/L) was much lower than initial FA concentration in non-irradiated solutions (226 µmol/L) (Fig. 2). Unlike UV treatment which showed an almost constant molar balance with time exposure (Akhtar et al., 1997), E-beam irradiation did not show a good agreement with the initial concentration of FA probably due to the presence of several other products which could not be accounted in the assay. This fact could be explained by the formation of volatiles compounds and/or the applied chromatographic conditions that did not allow quantification. The concentrations of pNBGA (≈23 µmol/L) and XA (≈20 µmol/L) increased until 1 kGy and 5 kGy, respectively. AHMP, PCA and pABGA were the least produced radio-products with individual concentrations lower than 10.0 µmol/L, independently of applied radiation doses (Fig. 2). In our study pABGA concentration remained almost constant over all radiation doses applied (Fig. 2). On the other hand, pNBGA concentration increased substantially up to 1 kGy, indicating that pNBGA was probably the leading radio-product in pABGA degradation pathway. Concerning PCA, its concentration reached 7.5 µmol/L when a 1 kGy radiation dose was applied to FA solutions. Nakken and Pihl (1966) studied the X-ray-induced damage to FA molecules and moieties in solution. They realized that X-ray-irradiated FA solutions (10^{-3} mol/L) also showed a slight increase in pABGA concentration (≈4.10^{-5} mol/L) up to a radiation dose of 6.10^{21} eV L^{-1}. These authors stated that in complex chemical and biochemical systems radiation-induced water radicals (i.e. hydroxyl radicals [•OH], aqueous electron [e^{-aq}], hydrogen atoms [•H], hydrogen molecules [H_2], hydrogen peroxide [H_2O_2], and hydrated protons [H_3O^+]) may react primarily at certain preferential sites. Our results are in agreement with these authors once radiation damage to FA was preferentially localized in the C_9–N_{10} linkage of FA. The increasing pNBGA formation compared to pABGA (up to 1.0 kGy) could suggest an initial FA breakdown followed by pABGA oxidation due to •OH radicals, highly mobile strong oxidants (WHO, 1994, 1999). Taking into account that the pH of an aqueous system can affect the end result of water radiolysis, the alkaline environment used in this study favored the aqueous electron formation (Molins, 2001). Alike hydrogen atoms (H•), solvated electron (e^{-aq}) is highly mobile and reductant which could initiate PCA reduction resulting in AHMP molecule.

PABA quantification was not achieved using the LC/UV method due both its very low concentration in irradiated FA solutions and also the not-resolved LC/UV chromatogram (Fig. 1). Thus, non-expressive FA radio-products were not quantified (Fig. 1).

Most of FA degradation products showed an increasing behavior mainly up to 1.0 kGy, although each radio-product exhibited different slopes (Fig. 2). On the other hand, this pattern was extended to pABGA and XA which showed increasing concentrations up to 3.0 and 5.0 kGy, respectively.

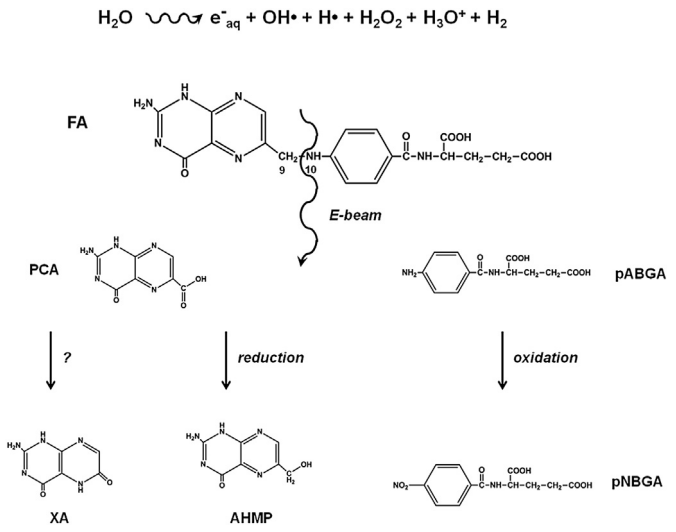

Fig. 3. Radiolysis of water and the proposed radio-degradation pathway of FA (folic acid). XA (xanthopterin); AHMP (6-(hydroxymethyl)pterin); PCA (pterine-6-carboxylic acid); pABGA (p-aminobenzoyl-L-glutamic acid); pNBGA (N-(4-nitrobenzoyl)-L-glutamic acid hemihydrate).

In view of these results, a probable mechanism of FA radiolysis could be delineated according to Fig. 3. E-beam processing lead to an initial FA cleavage yielding the radio-products PCA and pABGA. Oxidation and reduction reactions occurred during E-beam treatment resulting in different sub-products. A reduction step yielded PCA transformation into AHMP while an oxidation reaction transformed pABGA into pNBGA. Another non-elucidated reaction was responsible for XA formation from FA molecule.

4. Conclusions

Folic acid radio-degradation as a function of radiation dose implies vitamin losses. FA radiolysis lead to the formation of several by-products, most of them already found after usual food treatments (such as cooking, baking, microwave, freezing, etc.) and two radio-specific ones (pNBGA and AHMP) were identified. Care should be taken to the fact that this experiment was based in alkaline aqueous solutions of FA and degradation products standards, however it can be useful as a reference experiment for possible assessments of natural folates in fresh foods.

Acknowledgments

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