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Qualitative Determination of Photodegradation Products of Progesterone and Testosterone in Aqueous Solution

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Abstract

Direct photochemical degradations of progesterone (PR) and testosterone (TR), two naturally produced hormones, have been conducted in non-buffered aqueous solution (pH ranging between 5.5 and 6.0). The irradiation experiments were carried out in a batch reactor upon monochromatic (254 nm) and polychromatic (λ > 290 nm) at 25˚C. GLC/MS and LC/UV-DAD/MS analyses were performed to investigate phototransformation products after a solid phase extraction (SPE) step for analytes concentration. For each compound several by-products have been identified and are the same ones under both irradiation conditions. Because of the presence of the same chromophore (α, β-unsaturated group) absorbing UV radiations in both hormones, the majority of chromatographic peaks correspond to by-products formed according to identical mechanisms involving isomerization, enolization, oxidation and hydration to lead to the generation of lumiketone, cyclopentenone, spiro-hydration, oxidation and hydroxylation photoproducts.

Keywords

Photoproducts, Testosterone, Progesterone, GLC/MS, LC/UV-DAD/MS

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1. Introduction

The occurence of pharmaceuticals and hormones constitute an ongoing issue in many waters quality studies because they are more and more extensively and increasingly used in human and veterinary medicine, and also released continuously into the environment. Steroid hormones are classified as a group of endocrine disruptor chemicals (EDCs). An EDC is defined as "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of development processes [1]. These compounds have been described as causes of reproductive disturbance in humans and wildlife [2] [3] or susceptible to have adverse effects in human like declines in the quality, decreases in the quantity of sperm production, and the increase in incidences of certain cancers (prostate, testicular, breast, ovaries) [1]. However, it was clearly established that the diethylstilbestrol (DES), a synthetic estrogenic steroid, is responsible of infertility, spontaneous abortions in woman and genetic anomalies and the increases of risk of prostate and testicular cancers [4]-[6].

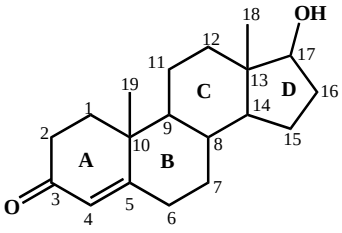
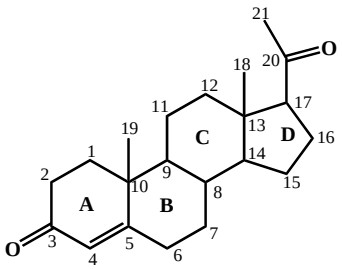
Natural estrogen (estradiol) and the synthetic one, ethinylestradiol, are known to have estrogenic effects even at weak concentrations. Then the lowest observable effect concentrations (LOEC) are 4.7 $ng{\cdot}L^{-1}$ for estradiol [7] and 0.17 $ng{\cdot}L^{-1}$ for ethinylestradiol [8].

This work is designed to study the photodegradation of two steroid hormones, testosterone and progesterone. They have similar cyclic ketone chemical structure with the ABCD rings system from C1 to C20 common to both compounds (**Table 1**).

Progesterone and testosterone are the primary natural progestin and androgenic hormones, respectively. In humans they have also various medical applications including hormone replacement therapy, contraception and palliative care for cancer treatment [13]-[15]. These hormones are mainly derived from the constant excretion (faeces and urine) of human and animals and are also released from sewage treatment plants, representing a risk for aquatic organisms and humans [16] [17].

The occurrence and the fate of testosterone and progesterone in the environment have been reported very little. It has been indicated that hormones are ineffectively treated in wastewater treatment plants (WWTPs) and sewage treatment plants (STPs) even if TR and PR were completely removed from aqueous phase in aerobic and anaerobic conditions using two pilot-scale municipal wastewater treatment plants [18]. Concentrations ranging from 0.3 to 0.8 $ng{\cdot}L^{-1}$ of testosterone were measured in the effluents of several WWPTs [19]. Similar concentrations were observed in a river with a maximum of 5.6 $ng{\cdot}L^{-1}$ [20]. Recently, TR was also detected at mean concentration of 9 $ng{\cdot}L^{-1}$ in hospital wastewaters [21]. Higher concentrations were detected in rivers and agricultural watershed, 16 $ng{\cdot}L^{-1}$ for TR and 12 - 14 $ng{\cdot}L^{-1}$ for PR [21]. In 2002, the analyses carried out in several

Table 1. Structure and physicochemical properties of testosterone and progesterone.

Hormones	Molar mass ($g{\cdot}mol^{-1}$)	Water solubility at 25˚C ($mg{\cdot}L^{-1}$)	pKa	Structures
Testosterone (TR)	288.4	24[a], 23.4[b]	17.4[d]	
Progesterone (PR)	314.5	7[c] - 8.81[b]	NA	

NA: not available. [a][9]; [b][10]; [c][11]; [d][12].

United States streams revealed maximum concentrations (median values) of progesterone and testosterone of 199 ng·L^{-1} (11 ng·L^{-1}) and 214 ng·L^{-1} (116 ng·L^{-1}) [22]. Up to (median values) 904 ng·L^{-1} (342 ng·L^{-1}) of PG and 635 ng·L^{-1} (258 ng·L^{-1}) of TR were detected in wastewater treatment works from South Africa [23]. They were present in groundwater with concentration in the range of 4.4 - 11.1 ng/L and 0.3 - 26.3 for ng/L PR and TR, respectively [24]. LOECs determined by Rosenmai *et al.* [25] were 3.1 µM for both steroid hormones.

Little is known about the photochemical transformation of testosterone and progesterone especially about the products formed from their direct photolysis. With regard to testosterone, photodegradation products have been identified in some studies [26]-[28]. To our knowledge, there has been no previous description study of PR photoproducts formation. However, photolysis implemented alone or in combination with catalysts in advanced oxidation processes is known to be one of the most effective techniques for removal of pharmaceuticals and other organic pollutant compounds from water [29]-[34].

This study focused on the identification of photochemical transformation products of progesterone and testosterone in purified dilute water. For both hormones, the direct photolysis has been performed under monochromatic ultraviolet light at 254 nm (the wavelength used for disinfection process in drinking water) and under polychromatic irradiation (λ > 290 nm) to simulate sunlight. Kinetic aspects and quantum yield determination were described in our previous work [35].

2. Materials and Methods

2.1. Chemicals

All chemicals were commercially products of the purest grade. Testosterone and progesterone were supplied by Acros organics (Noisy Le Grand, France). Methanol, sulphuric acid and acetic acid were from Carlo Ebra (Val-de-Reuil, France). Titanium (IV)-chloride and hydrogen peroxide (not stabilized) were provided by Sigma Aldrich (Lyon, France) and Fluka (Germany), respectively. Purified water from Millipore Milli-Q system (Millipore Milli RX75/Synergy 185, Millipore Corporation, France) was used to prepare all solutions.

2.2. Irradiation Experiments

The photolysis experiments at 254 nm and λ > 290 nm were conducted in two identical batch photoreactors. The volume of irradiated solution (thermostated at 25˚C) was 4 L and the optical pathlength was 6.75 cm. Lamps were located at the axis of reactor, in a quartz sleeve. A Vilbert Lourmat 6 W low-pressure mercury lamp was use for monochromatic irradiations. The flux of the lamp was evaluated using hydrogen peroxide as an actinometer as described elsewhere [36]. Typical values varying in the range 6.5 - 8.5 × 10^{-7} Einstein L^{-1}·s^{-1} were measured during the period of experiments. Polychromatic irradiations were performed with a Vilbert Lourmat 6 W low-pressure mercury lamp TM6. Due to their low solubility, testosterone and progesterone stock solutions (>20 µM) were prepared by stirring powders in purified water overnight and the undissolved compounds were removed by filtration through 0.45 µm Millipore HVLP membrane filter. Exact concentrations of solutions were determined by high performance liquid chromatography (HPLC) with UV detection at 245 nm and using calibration curves obtained from 100% methanolic solutions. Irradiated aqueous solutions of progesterone and testosterone ([PR]$_0$ = [TR]$_0$ = 10 µM) were prepared by dilution of stock solutions. All irradiation experiments were performed in three replicates.

2.3. Solid-Phase Extraction (SPE)

Irradiated solutions were extracted by SPE in order to increase parent products and their photoproducts with single-use 60 mg Waters oasis hydrophilic-lipophilic-balanced (HLB) cartridges based on the copolymer divinylbenzene-N-vinylpyrrolidone at a flow rate of 5 mL·min^{-1}. Then, 100 mL or 250 mL of solution was passed through SPE cartridges (the flow rate was about 5 mL·min^{-1}). The retained analytes were eluted with 2 mL of methanol and collected in a 4-mL chromatographic vial. This extract was analysed by LC/MS after dilution with 1 mL of water. For by GLC/MS analysis, the extract was evaporated to dryness under a gentle stream of nitrogen. The dry sample was reconstituted in 250 µL of methanol. This was repeated once for each irradiated solution.

The extraction yields for testosterone and progesterone were determined with concentration ranged from 1 to 10 µM and the mean recoveries values are 102% ± 2% and 104% ± 2%, respectively.

2.4. Chromatographic Analysis

The concentrations of stock solution of target compounds were measured by a HPLC coupled with a Waters 1525 binary pump, a Waters 717 autosampler and a Waters 2487 UV detector. A Hypersil BDS C18 column with particle size of 5 µm and pore size of 120 Å was used. The mobile phase was a mixture methanol/water (70/30, v/v) with a flow rate of 0.8 mL·min^{-1}.

The identification of by-products arisen from the phototransformation of the two compounds was performed after methanol SPE concentration by liquid and gas chromatography, both coupled to mass spectrometry. LC/UV-DAD/MS experiments were performed with a chromatographic system Thermo Surveyor equipped with an UV diode array detector (UV-DAD) giving the spectrum corresponding to the peaks and Thermo DECA XP Plus ion trap mass spectrometer. The mobile phase was a methanol/water (MeOH/H_2O) mixture with a flow rate of 0.3 mL·min^{-1}. The percentage of methanol linearly increased from 50% to 80% in 45 min and remained constant for 15 min. The analyses were carried out with an Uptispher C18 250 × 3 mm packed with spherical silica particles (size 5 µm and pore 120 Å) column from Interchim. The ionisation was performed in atmospheric pressure chemical ionisation (APCI) mode. The operating parameters were as follows: ion transfer capillary temperature 250˚C; vaporized temperature 450˚C; Sheath gas flow 13 L·min^{-1}; corona discharges 4 µA.

GLC/MS experiments were performed with a Hewlett-Packard HP 5890 Series II chromatograph/HP 5972 mass detector/HP 6890 autosampler. A capillary column AT-5MS from Alltech 30 m × 0.25 m (0.25 µm) was used. The injection temperature was maintained at 200˚C. The injected volume of samples was 1 µL in splitless mode (splitless time of 0.5 min). High grade helium was used as the carrier gas at a constant pressure of 53.1 kPa. The GC program was as follows: the column temperature was maintained at 50˚C, hold for 5 min and ramp at 50˚C min^{-1} to 150˚C, then ramp at 50˚C min^{-1} to 300˚C. Spectra were obtained in the total ion chromatogram (TIC) mode and scanning mode over the masse range of 30 - 650 amu (1.3 scan s^{-1}) for the identification of photoproducts.

Irradiated solutions were injected three times at variable time intervals of irradiation and the percentages of remaining hormones were calculated based on the initial concentrations.

3. Results and Discussion

For both hormones, many photoproducts were detected. Phototransformation experiments lead to the formation of the same photoproducts at 254 nm as at $\lambda > 290$ nm. Several of them have been identified by using simultaneously GLC/MS and LC/UV-DAD/MS, which give complementary information. Non-irradiated solutions of testosterone and progesterone were followed by both analytical techniques. The retention times were 20.1 and 40.0 min for TR and 21.4 and 53.0 for PR, respectively. Chromatograms did not show any presence of transformation products (peaks). Under our experimental conditions, the appearance of new peaks was observed at different irradiation times. Maximum photolysis times were 25 minutes for 254 nm irradiations (around 90% of degradation) and 24 hours for polychromatic light experiments (around 80% - 90% of degradation). Some examples of chromatograms of testosterone and progesterone are shown in **Figure 1** and **Figure 2**, respectively. GLC/MS chromatogram of TR was obtained after 15 min and 12 min for PR. LC/UV-DAD/MS chromatograms were recorded after 10 min and 12 min for TR and PR, respectively. The identification of photoproducts was carried out at these corresponding rates because all chromatographic peaks are present and are more intense. We have not been able to identify photoproducts corresponding to peaks labelled with question marks.

The ability of both analytical methods to separate peaks was measured by the determination of the resolution factor (Rs). The resolution between two peaks in a chromatogram is defined as the difference in retention times divided by the average of peak widths at the base [37].

In general, GLC/MS chromatograms show good resolution with peaks resolved completely ($Rs \geq 1.5$). Indeed, peaks with resolution factors greater than 1.0 can be accurately quantified [38]. However, low Rs values ($Rs \leq 1$) were obtained with peaks TR4/TR5 and TR7/TR8 for testosterone, and peaks PR4/PR5 for progesterone.

Using the LC/UV-DAD/MS system, the separation of many peaks of TR chromatogram (**Figure 1(b)**) became worse with Rs values ranging from 0.3 to 0.84 for peaks appearing before 25 min, between 25 and 30 min and between 42 and 47 min. For PR LC/MS chromatogram (**Figure 2(b)**), all peaks were well resolved with Rs greater than 1.3, except for peaks PR/PR13 ($Rs = 0.6$).

From these results, it appears that the best analytical conditions (mobile phase composition and conditions, temperature…) should be sought in order to improve the resolution of closely eluting compounds.

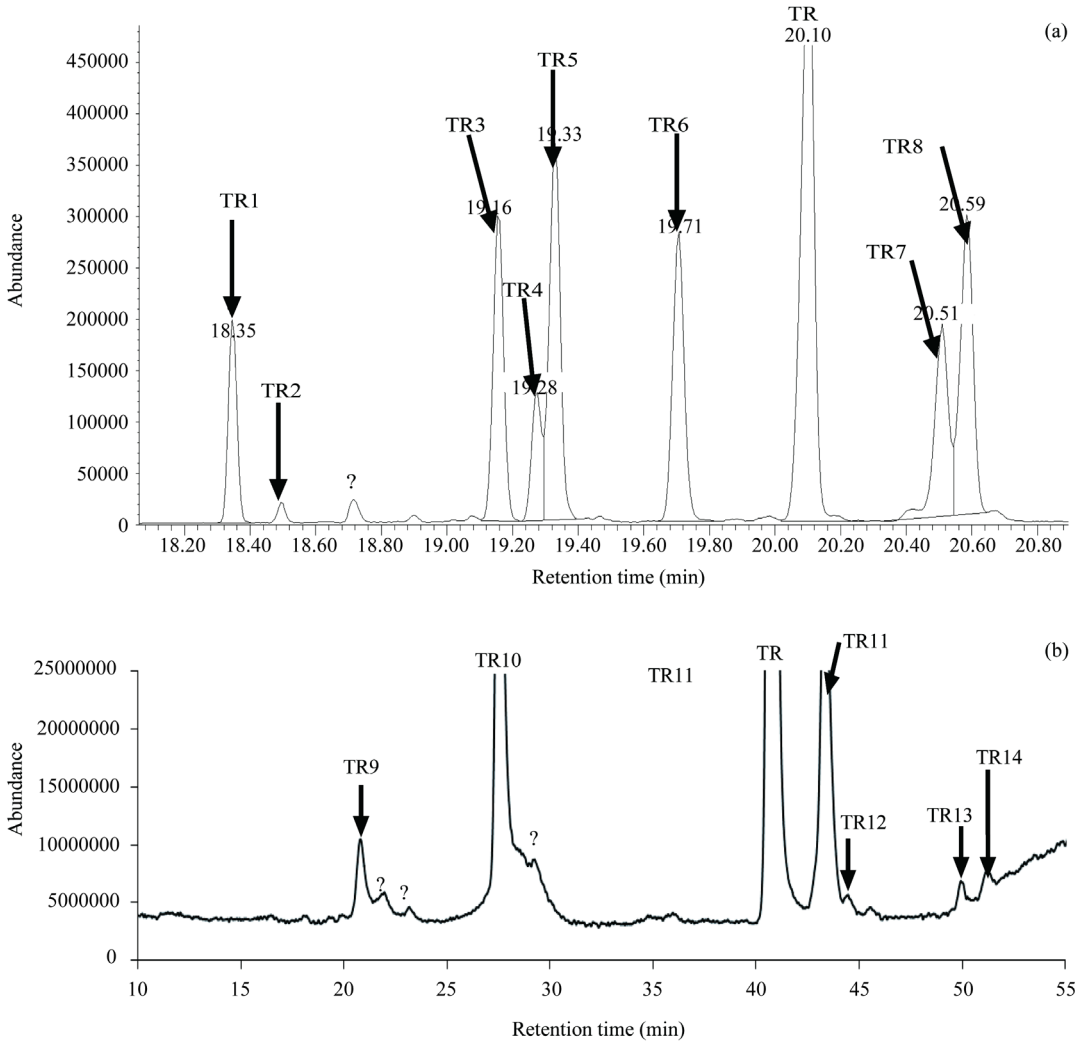


Figure 1. GLC/MS chromatogram of aqueous solutions of TR (a) ($[TR]_0$ = 10 µM, conversion rate of 80%) and LC/UV-DAD/MS chromatogram in APCI+ mode, (b) ($[TR]_0$ = 10 µM, conversion rate of 66%) irradiated at 254 nm and SPE concentrated.

3.1. Photoproducts with Mass Molecular Identical to That of TR (Peaks TR1 to TR6, TR11 and TR12) and PR (Peaks PR1 to PR6, PR12 and PR13)

Six GLC/MS peaks (TR1 to TR6 for testosterone and PR1 to PR6 for progesterone) have a molecular ion at 288 amu and 314 amu like the parent compounds. Only two were observed by LC/UV-DAD/MS (TR11-TR12 and PR12-PR13, respectively). In addition, mass spectra are very close (same shape and identical fragments) to those of TR and PR.

3.1.1. Peaks TR11 and PR12

The TR11 UV spectrum is similar to that of testosterone with an absorption band centred on 234 nm (244 nm for TR) consistent with a conjugated ketone. It is highly likely to match with the cyclopentenone photoproduct described by Vulliet *et al.* [39] and which has a UV maximum absorption at 236 nm. According to these authors it is formed through a photorearrangement of rings A and B by the homolytic cleavage of C1 - C10 bond with concomitant formation of the C5 - C10 double bond, followed by re-addition at C5 and cyclisation. An additional 5,6-bond migration occurs in the rearrangement mechanism. A similar process may take place for PR (with a single absorption band around 242 nm). Thus, the proposed structures of TR11 and PR12 are illustrated in **Figure 3**.

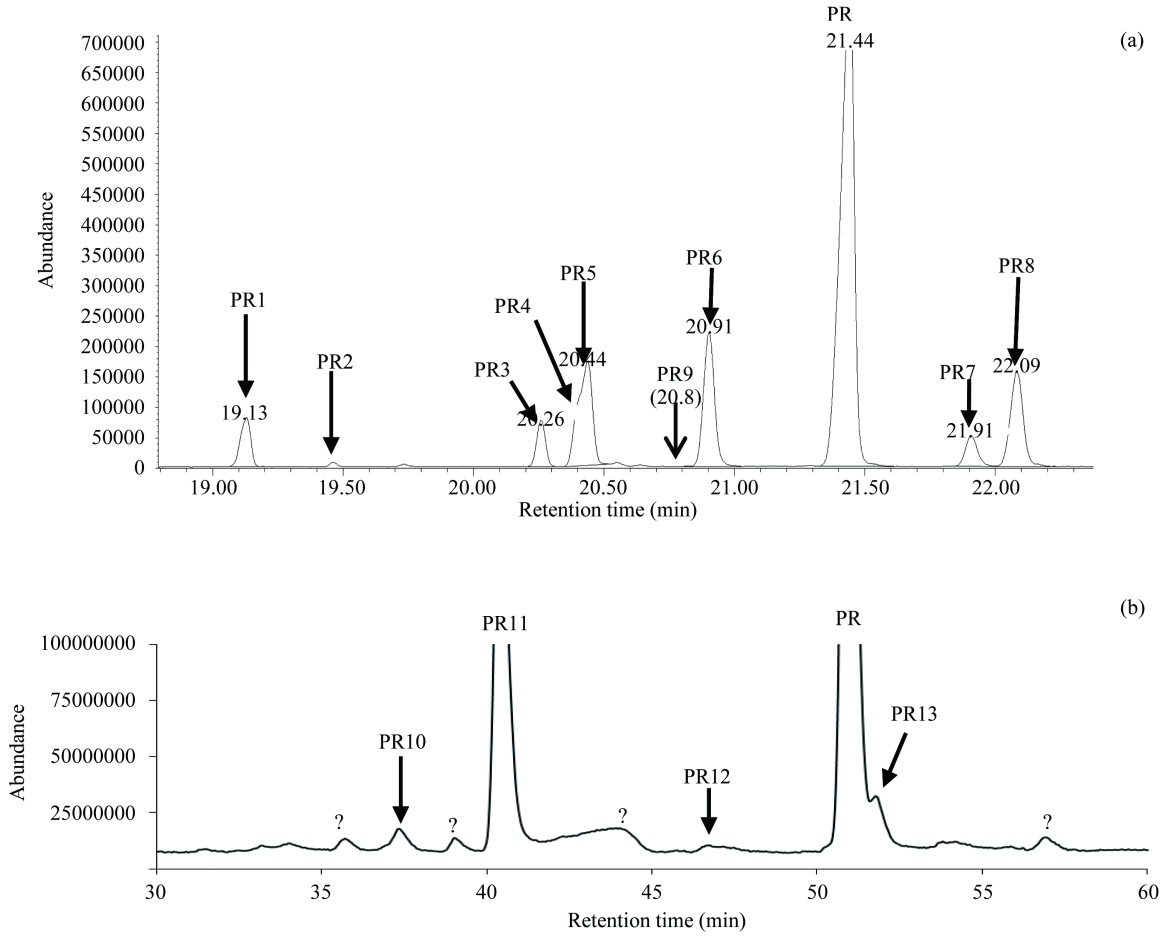


Figure 2. GLC/MS chromatogram of aqueous solutions of PR (a) ($[PR]_0$ = 10 µM, conversion rate of 56%) and LC/UV-DAD/MS chromatogram in APCI + mode, (b) ($[PR]_0$ = 10 µM, conversion rate of 57%) irradiated at 254 nm and SPE concentrated.

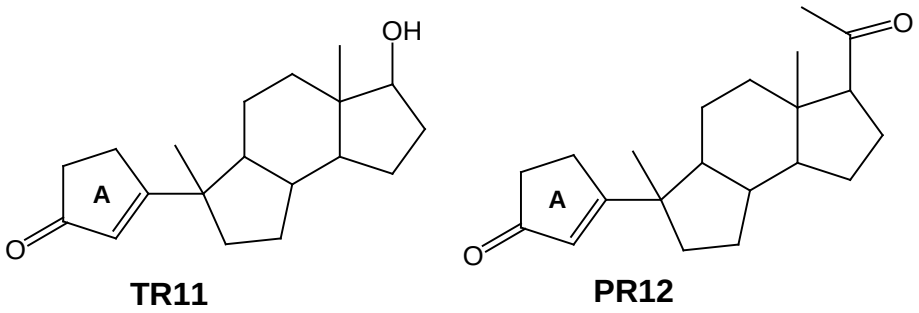


Figure 3. Proposed structures for photoproducts TR11 and PR12.

3.1.2. Peaks TR1 to TR6, TR12 and PR1 to PR6, PR13

The photoproduct associated with the peak TR12 (detected by LC/UV-DAD/MS) presents two main absorption bands. The first one is located at 204 nm. The second is very broad with an absorption maximum around 243 nm and extending to 330 nm that is a characteristic of enolic structures. Therefore, the corresponding compounds may be one of four photoenols may be formed according to the configuration Z/E of the double bonds in ring A of testosterone (photoproduct PR13 for progesterone) (**Figure 4(b)**). Indeed, ketone α, β-unsaturated compounds may be involved in isomerization Z/E and enolization reactions by photochemical activation of double bonds

[40] [41]. Therefore, by combining these reactions, it is possible to form five photoproducts of the same molecular mass as TR and PR. Thus, each of the six chromatographic peaks (TR1 to TR6 for TR and PR1 to PR6 for PR) can represent either the photoisomeric Z/E product of TR and PR (**Figure 4(b)**), either one of four photoenols (**Figure 4(a)**). Thereby, lacking data from LC/MS experiments, it is conceivable that TR1, TR2, TR3, TR4, TR5 or TR6 corresponds to the (1,5,10)-cyclopropyl-17β-hydroxyandrostane-2-one (m/z = 288) which was proposed by Vulliet *et al.* [27] as a luminketone rearrangment product (only ring A) of the direct photolysis of TR at 254 nm and 313 nm in water (**Figure 4(c)**) or the cyclopentenone product (described above) (**Figure 4(d)**). By analogy with TR, proposed structures for products from PR1 to PR6 are detailed in **Figure 4**.

We are not able to explain the difference between the number of chromatographic peaks detected by GLC/MS and LC/UV-DAD/MS. Additional work is needed, firstly to understand this phenomenon, and also to assign a chemical structure for each.

3.2. Photoproducts with +14 Amu with Respect to TR (Peak TR7) and PR (Peak PR7)

Mass spectra corresponding to the peak TR7 and PR7 present molecular peaks at 302 (100%) 328 and (100%) *i.e.*, +14 amu compared to PR and TR, respectively. These photoproducts have been detected only by LC/MS analysis. For PR, the spectrum also shows a first fragment at 313 and 285 amu being able to correspond to the

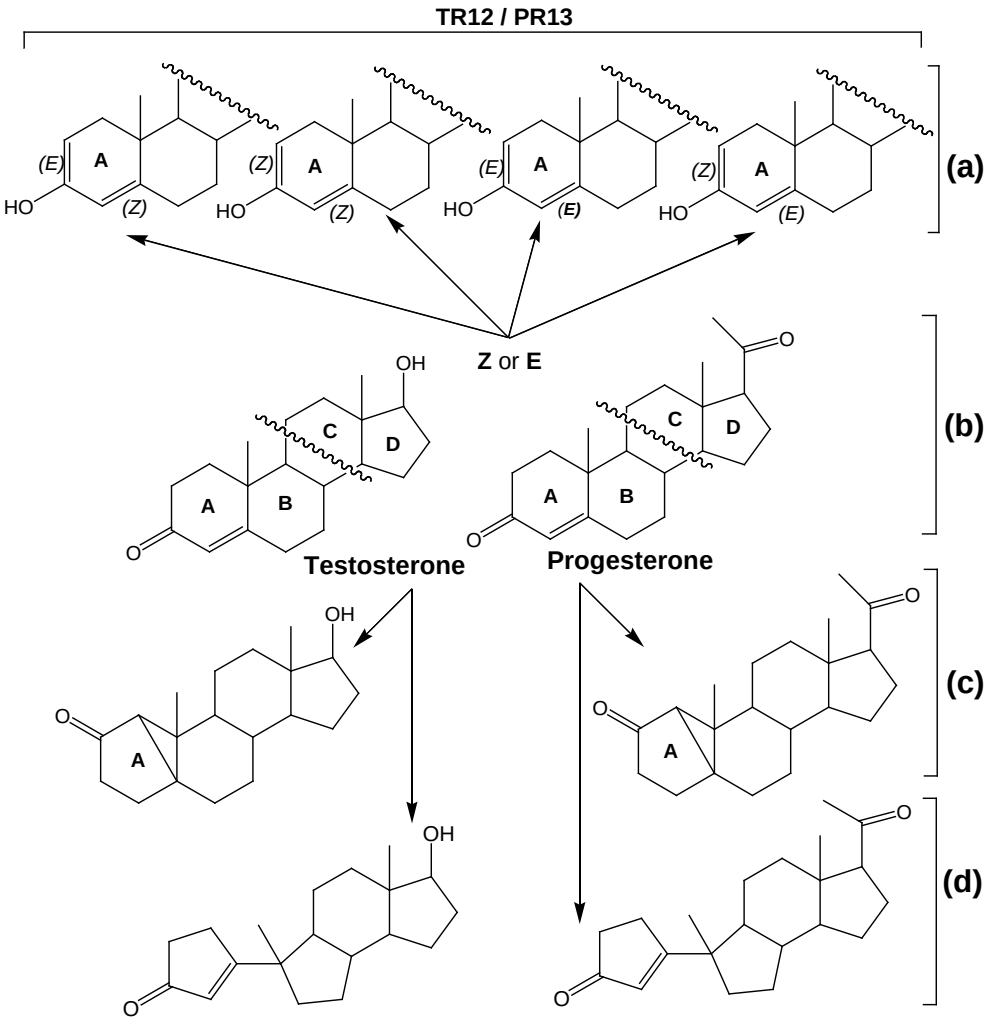


Figure 4. Proposed products structures in the direct photochemical degradation of testosterone [(a): Possible structures for TR12—(from (a) to (d)): Possible structures for TR1, TR2, TR3, TR4, TR5 and TR6] and progesterone [(a): Possible structures for PR13—(from (a) to (d)): Possible structures for PR1, PR2, PR3, PR4, PR5 and PR6].

losses of CH_3 (−15 amu) and CH_3-CO (−43 amu), respectively. According to TR, the first fragment corresponds to a loss of −15 amu. The corresponding structures proposed for TR7 and PR7 would result from an addition of an oxygen atom follow by an oxidation as proposed in **Figure 5**.

3.3. Photoproducts with +18 Amu with Respect to TR (Peak TR8) and PR (Peak PR8)

Peaks PR8 and TR8 are hydration by-products (+18 amu) show mass spectra with molecular ions at 332 and 306 amu with respect to PR and TR. Fragments at 314 amu (for PR) and 288 amu (for TR) may result from PR and TR, respectively. For testosterone, this peak may correspond to a hydroxylation at the C5 position of TR as described after a water-methanol (80/20, v/v) photolysis at 254 nm [26] (see in **Figure 6(a1)**). A similar way of reaction is possible for the progesterone which has the same main chromophore (α, β-unsaturated function) responsible of their light absorption. It is also possible that this by-product comes from the hydration of an enol form leading to structures proposed in **Figure 6(a2)**. No peak corresponding to this photoproduct has been detected by LC/MS. According to Vuillet *et al.* [28] and based on nuclear magnetic resonance (NMR) characteristics, TR8 could be the major product they found to be a spiro-compound (**Figure 6(b1)**). On this basis, the structure in **Figure 6(b2)** can be proposed for PR8.

3.4. Other Photodegradation Products of Testosterone

3.4.1. Peaks TR10, TR13 and TR14

LC/MS analyse shows peaks TR10, T13 and T14 with identical mass spectrum in the APCI positive mode with base peaks at 271.6 amu (100%), 271.3 amu (100%) and 271.4 amu (100%), respectively. This could be the dehydration of the ion fragment at 289 amu as observed for TR. However, due to the high intensity of the peak, it could be envisaged that it corresponds to a dehydration photoproduct of testosterone on the carbon in position 17 we proposed for the estradiol [42] as given in **Figure 7**.

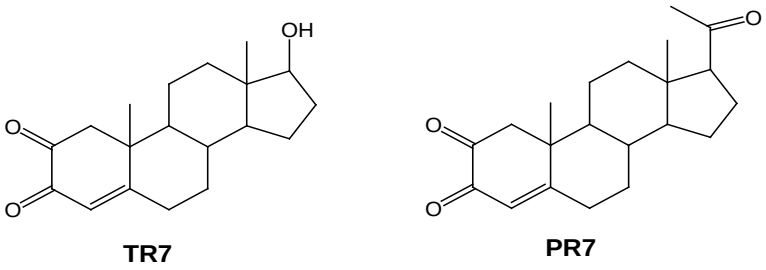


Figure 5. Proposed structures for photoproducts TR7 and PR7.

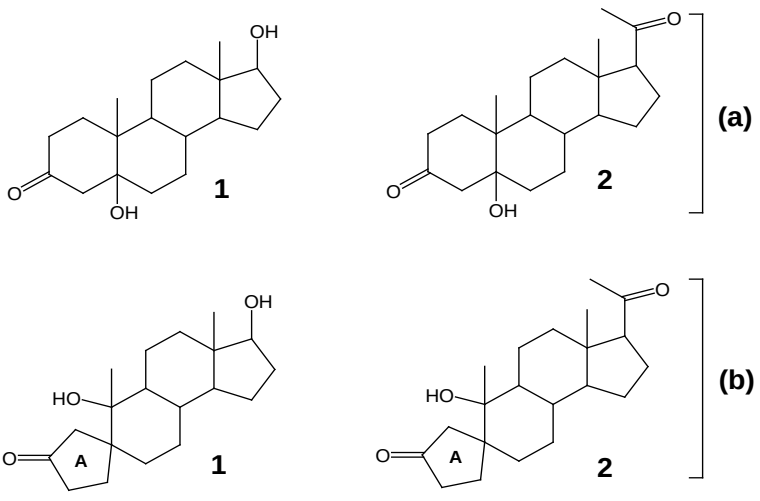


Figure 6. Proposed structures for photoproducts TR8 (a1 and b1) and PR8 (a2 and b2).

3.4.2. Peak TR9

For photoproduct TR9, no chemical structure could be proposed. Several fragments are observed: 321.1 (100%), 303.2 (94%), 335.0 (73%), 289.2 (47%) and 285.2 amu (29%). Unfortunately, the APCI negative mode did not provide additional information because of absence of response. This could be probably due to the low chemical structure stability in the APCI source.

3.5. Over Photodegradation Products of Progesterone

3.5.1. Peaks PR9 and PR10

The peak PR9 is observed during the GLC/MS analysis of an irradiated solution with a conversion rate of 85% after a SPE concentration. It is not quite visible on the chromatogram of the **Figure 2(a)** indicating that it is probably a secondary photoproduct. The mass spectrum presents a molecular ion at 346 amu (10%) with a first fragment at 328 *i.e.*, −18 amu corresponding to his dehydration. PR10 LC/MS chromatograms revealed the molecular peak at 345.1 amu in positive APCI mode. The APCI negative mode showed three intense peaks at 329.2, 347.1 and 361.1 amu. So it is reasonable to consider that the molecular ion at 347.1 amu is closely related to the ion 345.1 amu in the APCI-ion mode. This would correspond to +32 with respect to progesterone. In addition, the UV spectrum of the peak PR10 (m/z = 346) exhibits an absorption band around 272 nm which is characteristic of carbonyl functions. The proposed structure (**Figure 8**) corresponding to photoproducts PR9 and PR10 has been identified in progesterone water ozonation with an open cycle at carbone C4 [43].

3.5.2. Peak PR11

In the case of the peak PR11, a LC/MS mass spectrum similar to that of PR was obtained with a base peak at 315.3 amu (100%) and a other peak at 316.3 (20%) in positive ionisation mode. The MS/MS fragmentation of these ions gave ions at 297.1 (100%) - 279.4 (20%) and 297.1 (73%) - 279.3 (18%), respectively. In negative mode, two ions were detected, 332.1 (100%) and 333.3 (34%). Their MS/MS fragmentation gave primarily ion at 315 amu. According to these results, no chemical structure could be proposed.

4. Conclusion

This paper presents an investigation of testosterone and progesterone under UV phototransformation in aqueous solution. Many photoproduct structures have been proposed by using LC/MS and GLC/MS analyses. Some of them were obtained through the complementary data provided by both devices. By-products identified at 254 nm were also identified at $\lambda > 290$ nm. For several of them the mechanisms of formation for TR are identical to those of PR and involve photoisomerization, photoenolization, oxidation and hydration to lead to the generation of lumiketone, cyclopentenone, spiro-hydration, oxidation and hydroxylation photoproducts. The majority of

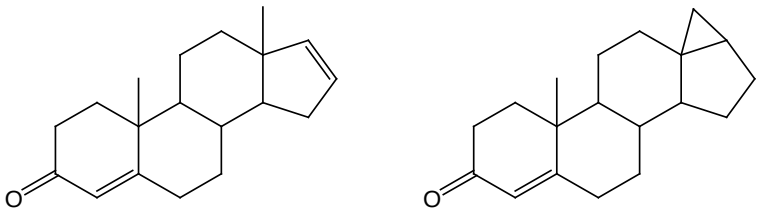


Figure 7. Proposed structures for photoproducts TR10, TR13 and TR14.

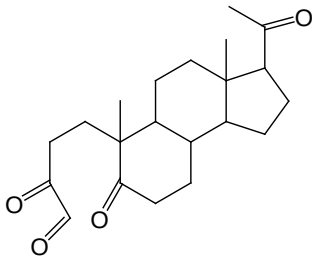


Figure 8. Proposed structures for photoproducts PR9 and PR10.

these photoproducts have identical molar masses as parent compounds and are easily observed by GLC/MS. This is probably due to a weak response or a low concentration to allow their HPLC/UV/MS/detection. For each of the steroid hormones, a photoproduct with +14 amu and two structures with +18 amu with respect to the parent compounds have been proposed. For testosterone, a C17 dehydration product formation was suggested while for progesterone an open cycle photoproduct seemed to be formed. To our knowledge, the by-products proposed for progesterone and some of testosterone photoproducts in this work are described for the first time in water photolysis studies maybe because of the low water solubility of these hormones.

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