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Kinetic and mechanistic investigations of progesterone reaction with ozone

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ABSTRACT

The removal of progesterone by ozone in aqueous solution was studied in this work. The absolute rate constant was evaluated and first by-products were identified.

The reaction was studied in the 2.0–8.0 pH range and was found to be a second-order reaction, first-order relative to each compound concentration. The rate constant, determined by kinetic experiments in presence of an OH radical scavenger (tert-butanol), was independent of pH. The value was evaluated to be equal to $480 \pm 30\,M^{-1}\,s^{-1}$ by two kinetic methods.

Mass spectrometry analyses were performed to investigate primary degradation products generated by the reaction of ozone with progesterone. Two by-products were evidenced. According to these results, a degradation pathway of progesterone reacting with ozone was proposed.

1. Introduction

An endocrine disruptor compound (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 developmental processes" (Kavlock et al., 1996).

As time goes on, the occurrence of molecules owning these properties in the aquatic environment has a growing concern (Richardson, 2003). This is largely explained by the potential effects of EDCs on health. Actually, several studies have reported abnormalities in wildlife exposed to EDCs in vitro but also in situ (Kavlock et al., 1996; Davis et al., 1999; Levi, 1999; Sedlak et al., 2000). In Human, diethylstilbestrol, a synthetic oestrogen, has been proved to cause cancer via an endocrine disruption mechanism (Herbst et al., 1971). In the same way, other examples of sporadic cases have been described in specific situations of Human beings exposed to high EDCs (Jacobson and Jacobson, 1996; Mocarelli et al., 1996; Aoki, 2001).

Moreover, Human is exposed by several ways to chemicals (absorption of contaminated water or contaminated food with molecules such as pesticides, leaching compounds from packaging materials, absorption of medicines…) which lead to the increase of the complexity of the problem (Levi, 1999).

Today, EDCs are suspected to be responsible of reproduction abnormalities such as uterus and ovaries diseases, spontaneous abortions, sperm quality declines, certain cancers incidences increases (breast, prostate, testicular) … (Kavlock et al., 1996).

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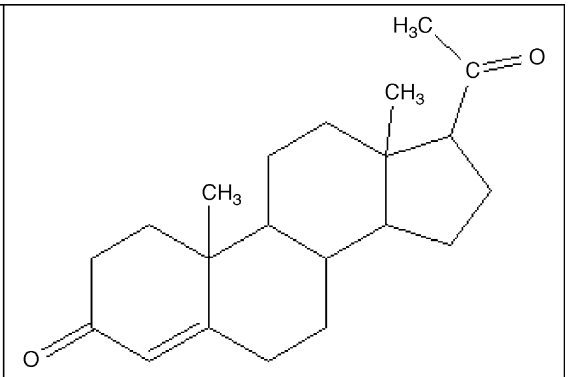
Progesterone : pregn-4-ene-3, 20-dione $C_{21}H_{30}O_2$ F.W: 314.5 $gmol^{-1}$ No pKa Reproductive hormone	

Fig. 1 – Structure and physicochemical properties of progesterone.

The global aim of our work is to study the fate of EDCs during drinking water production. Among processes used for disinfection, chlorination or ozonation are the most largely used.

Because of their own nature of hormone, natural hormones figure among the EDCs list. Previous studies generally focused on steroid reproductive hormones (such as estradiol, estrone, estriol...) because of their environmental abundances (Huber et al., 2003; Ternes et al., 2003). To complete the knowledge of the behaviour of natural hormones during water treatment, we performed a complete study of progesterone ozonation. The structure and characteristics of this steroid molecule are presented in Fig. 1. Little is currently known about this compound, however its environmental abundance is significant. Actually, Koplin et al. (2002) detected the presence of progesterone in 4.3% of 139 United States streams and a mean concentration of 0.11 $\mu g L^{-1}$ (maximum 0.2 $\mu g L^{-1}$) was measured.

A previous work concerning chlorination kinetics of steroid hormones has shown that, contrary to aromatic ring containing hormones (estradiol, estrone, estriol), progesterone did not react with chlorine at pH between 3.5 and 8.5 even in the presence of a large excess of chlorine (Deborde et al., 2004).

To complete this issue, we studied the oxidation of progesterone with ozone, which is a more oxidizing agent than chlorine.

In a first step, absolute rate constant was determined by kinetic experiments with and without OH radical scavenger. Then, the mechanism was investigated with the identification of first by-products by mass spectrometry.

2. Materials and methods

2.1. Standards and reagents

Progesterone was supplied by acros organics (purity $\geqslant$98%) and all reagents used were of analytical purity. Purified water (18 MΩ cm) was produced with a Millipore apparatus. Stock solutions of sodium thiosulfate (32 mM), phosphate buffer (500 mM), tert-butanol (100 mM) were prepared by dissolution in water of the commercial compound. Aqueous solution of progesterone ($\approx$ 20 μM) was prepared by stirring the powder in water for two days and the undissolved compound was removed by filtration over a 0.45 μm filter. The exact concentration was determined by high-performance liquid chromatography (HPLC) and UV detection (250 nm) after calibration with methanolic solution of product.

2.2. Ozonation

A Trailigaz labo 76 ozone generator (set at 40 W) produced ozone from oxygen. The ozone was continuously dissolved in stirred water (cooled by a cryostat RC 20, CS Lauda set at 4 °C).

2.3. Kinetic experiments

Kinetic experiments were performed by monitoring progesterone degradation in the presence of an ozone excess. In order to limit losses of ozone, batch reactors and glass syringes of low sizes (respectively, 100 mL and 5–20 mL) were used. The flasks were kept closed during all the experimental times.

In final, batches contained approximately 20 μM of aqueous ozone, 1 μM of progesterone, pH adjustment reagent and 10 mM of tert-butanol when required. Tert-butanol was added to inhibit OH radical reactions generated by ozone decomposition (Legube, 2003; Von Gunten, 2003). pH values were adjusted with sulphuric acid for pH $\leqslant$ 5.0 and with phosphate aqueous buffer for pH > 5 (final phosphate concentration = 5 mM). Kinetic runs were initiated by injecting, under rapid mixing, the amount of progesterone stock solution in batches containing ozone. Aliquots of 0.8 mL were withdrawn ($n = 9$) from the batch thermostated at 18 $\pm$ 1 °C and were transferred into vials containing 20 μL of sodium thiosulfate (32 mM) to quench the residual ozone and stop the oxidation reaction. In all cases, ozone concentration was measured before and after reaction. Temperature and pH were also controlled at the end of experiments. Blanks were performed in a same manner with water instead of ozone solution.

2.4. Analytical methods

2.4.1. Ozone determination

The Indigo method was used for the determination of dissolved ozone concentration in water. Stock solution was prepared as previously described (Bader and Hoigne, 1981; Standard methods for the examination of water and wastewater, 1995). The measurement of the absorptivity was performed at 600 nm with a spectrophotometer SAFAS UV-Visible 320.

2.4.2. Hydrogen peroxide determination

The titanium(IV) chloride method was used to determine H_2O_2 concentration (Eisenberg, 1943). Absorptivity was measured at 410 nm in a 10 cm cell ($\varepsilon = 700\,M^{-1}\,cm^{-1}$) with a spectrophotometer Secoman S 1000. The detection limit was evaluated to be about 1.7 μM.

2.4.3. Kinetic experiments

HPLC analyses were performed at room temperature ($24 \pm 2\,^{\circ}C$). A Waters 717 plus autosampler, a Waters 600E pump, a C_{18} column Kromasil (4.6 mm internal diameter, 25 cm length) packed with 5-μm spherical particles were used. The mobile phase was a mixture of methanol/water 80/20 used at $1\,mL\,min^{-1}$. The injected volume of sample was 200 μL. Column effluent was monitored with a UV-visible spectrophotometer (Waters 2487) at 250 nm (retention time of progesterone: 9.5 ± 0.5 min).

2.4.4. By-products identification experiments (LC/MS and MS^2)

Samples were analysed with a chromatographic system thermo electron surveyor equipped with a diode array detector (thermo electron) and an ion trap mass spectrometer DECA XP max (thermo electron). Atmospheric pressure chemical ionisation (APCI) was selected to provide a better ionisation of progesterone both in positive and negative detections.

HPLC analyses were carried out with a Uptispher HDO C_{18} column (3 mm internal diameter, 25 cm length) packed with 5-μm spherical particles. A methanol/water mixture was used as the mobile phase 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.

Direct injections of progesterone aqueous solutions were performed to optimise mass spectrometer parameters. The following parameters were then used: vaporiser temperature (450 °C), sheath gas flow rate (80 a.u.), auxiliary gas flow rate (5 a.u.), corona discharge (4 μA) and ion transfer capillary temperature (250 °C).

3. Results and discussion

3.1. Determination of stoichiometric factor

For following experiments, the stoichiometry of reaction was supposed to be one mole per mole. According to this hypothesis, the amounts of ozone necessary to oxidize between 20% and 100% of progesterone were added to the reaction bottles. The experiments were conducted in presence of tert-butanol 10 mM. The remaining progesterone concentration of each reactor was measured by HPLC analyse after all ozone had reacted.

The stoichiometry was defined as the ratio $\eta = n_{ozone}/n_P$ where n_{ozone} was the amount (in μmole) of consumed ozone and n_P was the quantity (in μmole) of disappeared progesterone. The results are presented in Table 1. Accordingly, a stoichiometry of 1.03 ± 0.13 (RSD = 12%) was obtained. Therefore, reaction between progesterone and ozone could be described by a mole to mole reaction.

3.2. Determination of rate constant reaction

Progesterone ozonation was considered as a second-order reaction with first-order relative to progesterone [P] and to ozone [O_3] concentrations, and with a stoichiometry of one mole per mole.

The rate of progesterone disappearance could be formulated as:

$$v = -\frac{d[P]}{dt} = k[P][O_3], \quad (1)$$

where k was the second-order rate constant.

Table 1 – Calculation of stoichiometry's reaction

$[O_3]_0$ (μM)	$[P]_0$ (μM)	% of disappeared progesterone	n_P (μmole)	n_{ozone} (μmole)	Stoichiometry
2.8	10.6	29	0.31	0.28	0.91 ± 0.07
5.6	10.6	57	0.61	0.56	0.93 ± 0.07
8.5	10.6	85	0.89	0.85	0.95 ± 0.07
11.3	10.6	100	1.05	1.13	1.07 ± 0.08
5.0	19.3	20	0.42	0.55	1.30 ± 0.13
10.0	19.3	48	1.02	1.10	1.08 ± 0.10
15.0	19.3	74	1.57	1.65	1.05 ± 0.10
17.4	19.3	92	1.95	1.92	0.98 ± 0.10
				Mean result	1.03 ± 0.13
					RSD 12%

$[O_3]_0$ and $[P]_0$ were, respectively ozone and progesterone initial concentrations; % was the percentage of progesterone degradation after all ozone had reacted; n_P was the amount (in μmole) of disappeared progesterone; n_{ozone} was the amount (in μmole) of consumed ozone; stoichiometry was defined as the ratio $\eta = n_{ozone}/n_P$.

Table 2 – Rate constants obtained as a function of pH and calculation methods

pH	Temperature ±1 °C (°C)	Apparent first-order method k ($M^{-1} s^{-1}$)	Second-order method k ($M^{-1} s^{-1}$)
2.00	18.0	485 ± 62	518 ± 23
3.53	19.0	487 ± 40	521 ± 15
4.87	18.5	460 ± 53	496 ± 14
6.49	18.0	444 ± 54	457 ± 09
7.96	17.0	nd	444 ± 11
Mean	18 ± 1	469 ± 21	487 ± 36
Relative standard deviation		4.5%	7.0%
Student's test (0.05 risk)		No differences	

nd = not determined.

Under certain experimental conditions, ozone was in large excess and its consumption was checked to be enough low i.e. decrease in ozone concentration lower than 20% (David Yao and Haag, 1991). Hence, ozone concentration could be considered constant during reaction and the expression of the rate of progesterone degradation became:

$$v = -\frac{d[P]}{dt} = k_{obs}[P], \quad (2)$$

where $k_{obs} = k[O_3]$ = apparent first-order kinetic constant.

Therefore, a plot of ln [P] as a function of the reaction time led to a straight line from which k_{obs} could be determined. The rate constant of reaction (k) was calculated from the following equation:

$$k = k_{obs}/[O_3], \quad (3)$$

where $[O_3]$ was the initial ozone concentration i.e. $[O_3]_0$.

Under other experimental conditions, particularly for experiments at pH 8.0, ozone depletion was too high to assume a constant ozone concentration. Then, integration of Eq. (1) yielded:

$$kt = \left[\frac{1}{[P]_0 - [O_3]_0}\right] \ln \frac{[P][O_3]_0}{[P]_0[O_3]}, \quad (4)$$

where $[P]_0$ and $[O_3]_0$ were, respectively progesterone and ozone initial concentrations and [P] and $[O_3]$ progesterone and ozone concentrations for a given time.

Under these experimental conditions, measurements of $[O_3]$ could not be achieved because reactions were too fast. Hence, the stoichiometry of the reaction (cf. 3.1.) was used together with the initial ozone concentrations to calculate the concentration of ozone at different times. Therefore, these values were injected with [P], $[P]_0$, $[O_3]_0$ experimentally measured in Eq. (4) to determine the value of k.

The calculated rate constants according to the two described methods are gathered in Table 2.

The pH values did not influence the rate constant. This observation is well justified by the fact that progesterone does not present any acid or basic character and that only ozone reacted (OH radicals seem to be very well scavenged by tert-butanol).

The plot of k_{obs} obtained by the apparent first-order calculation method as a function of the initial ozone concentration is presented in Fig. 2. A straight line was obtained with a correlation coefficient higher than 0.99 confirming the second order of the reaction.

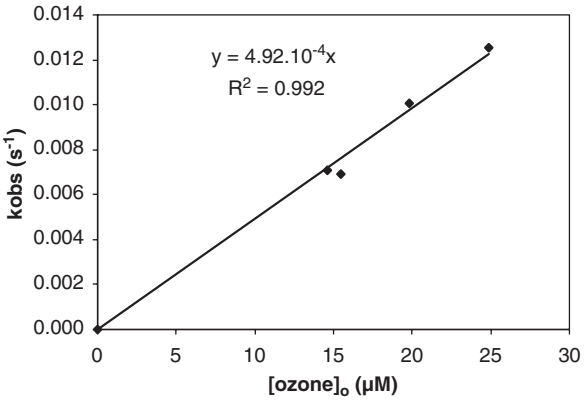


Fig. 2 – Straight line $k_{obs} = k\,[ozone]_0$ for the different experiments.

Table 3 – Rate constants as a function of pH with or without tert-butanol

Second-order method k ($M^{-1} s^{-1}$)	Mean pH = 3.54	Mean pH = 7.94
With tert-butanol	521 ± 15	444 ± 11
Without tert-butanol	575 ± 52	814 ± 31

The calculation method does not significantly influence the rate constant. The mean value is equal to $480 \pm 30\,M^{-1} s^{-1}$, which is far lower than the rate constant defined for steroid phenolic hormones ($10^6\,M^{-1} s^{-1}$) (Huber et al., 2003; Deborde et al., 2005). The activated aromatic systems are actually known to exhibit higher reactivity towards ozone (Legube, 2003; Von Gunten, 2003).

From this rate constant, the half-life time of progesterone in water was evaluated to be about 1 min under conditions closed to those used in drinking water production process (ozone concentration about $1\,mg\,L^{-1}$ i.e. 21 µM).

Similar experiments were performed without OH radical scavenger. The rate constants calculated with second-order calculation method are gathered in Table 3. At acidic pH, no

significant influence of tert-butanol on the rate constant was observed. On the contrary, a significant enhancement of the rate constant was observed at higher pH in the absence of OH radical scavenger. This is related to the reactions of OH radicals arising from ozone decomposition (Legube, 2003; Von Gunten, 2003), highly reactive species certainly able to accelerate the degradation of progesterone. The tert-butanol addition at basic pH inhibited the oxidation by OH radicals and led the rate constant value comparable to the one obtained at acidic pH.

3.3. *By-products identification*

Aqueous progesterone solution (19.2 μM) was directly injected in the mass spectrometer to optimise ionisation source parameters. Progesterone (FW = 314.5 g mol^{-1}) could be detected in negative and positive modes, respectively at m/z = 313 uma ($M - H^-$) and m/z = 315 uma (MH^+). The parameters of the mass spectrometer were optimised to obtain the largest abundance of these ions in the two ionisation modes as already mentioned.

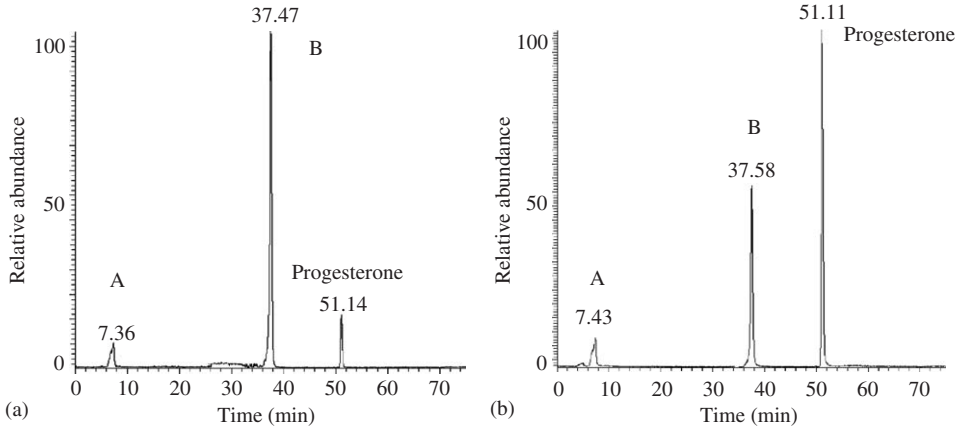


Fig. 3 – Mass spectrometry chromatograms of aqueous ozonised progesterone solution (48% of degradation): (a) negative APCI mode; (b) positive APCI mode.

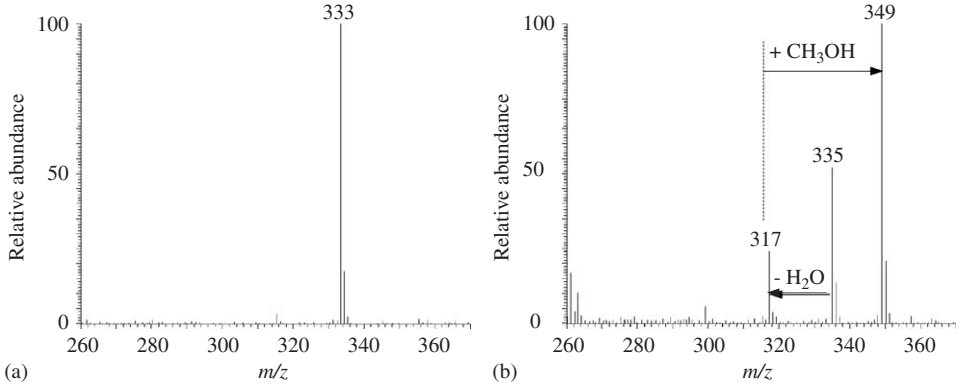


Fig. 4 – MS spectra of A: (a) negative APCI mode; (b) positive APCI mode.

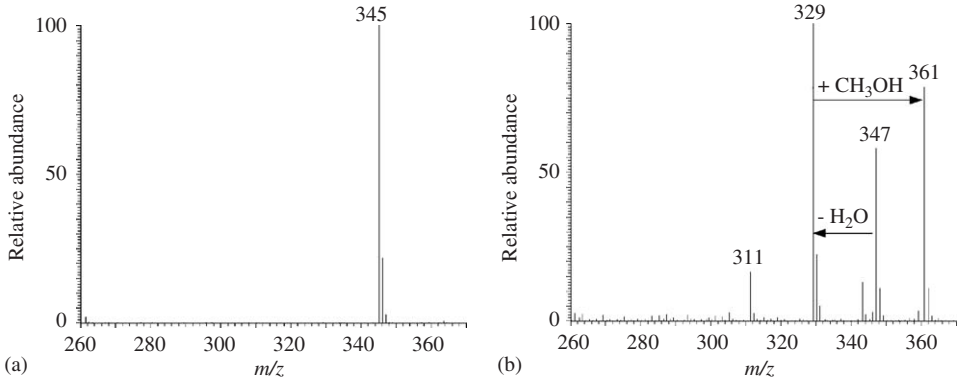


Fig. 5 – MS spectra of B: (a) negative APCI mode; (b) positive APCI mode.

Table 4 – Main mass fragments of progesterone and A and B by-products obtained by means of LC/MS or LC/MS^2 in negative and positive APCI modes

Products	Negative mode: m/z values (relative abundance)		Positive mode: m/z values (relative abundance)	
	MS	MS^2	MS	MS^2
A	333 (100)	261 (100) 289 (15) 315 (2.5)	317 (25) 335 (52) 349 (100)	281 (35), 299 (100) 317 (100), 299 (10) 317 (100), 299 (7)
B	345 (100)	261 (100) 317 (5)	329 (100) 347 (58) 361 (78)	311 (100), 293 (34) 329 (100), 311 (30) 343 (100), 333 (14) 311 (20)
Progesterone	313 (100)	285 (22) 295 (100)	315 (100)	297 (100) 279 (28)

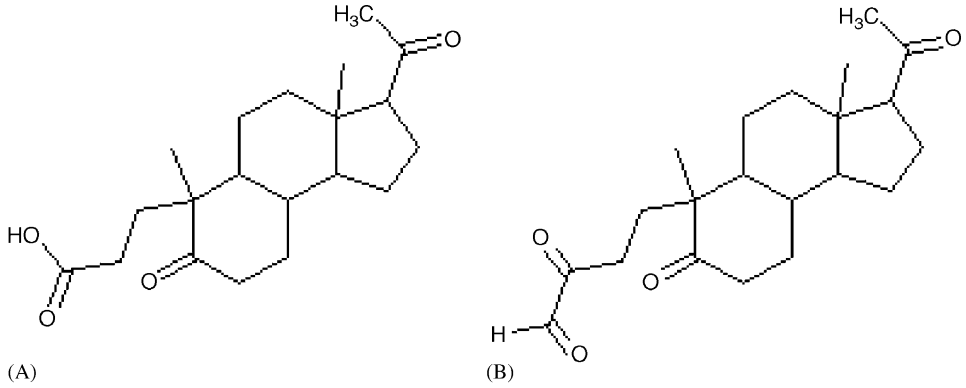


Fig. 6 – Proposed structures for A and B products.

Samples of aqueous ozonised progesterone were analysed by HPLC–MS to determine by-products. Chromatograms (Fig. 3) of the ozonised progesterone products analysed by LC/MS revealed two additional peaks compared to that of sample with unreacted progesterone (chromatogram not shown). The peaks (labelled A and B) observed on chromatogram (Fig. 3) at 7 and 37 min correspond to two by-products more polar than progesterone. It had to be noted that the response of the products was different according to the ionisation mode selected. The corresponding MS spectra are presented in Figs. 4 and 5 both in negative and positive ionisation modes. The ions obtained in the first stage MS analyses have been isolated in the ion trap and MS^2 experiments were performed. The overall results are gathered in Table 4.

The following observations could be noted:

- MS experiments

  Progesterone: It was detected in negative ($M-H^-$: $m/z=313$ uma) and positive (MH^+: $m/z=315$ uma) modes. The ionisation seemed to be more efficient in positive than in negative mode.

  Both degradation products: They were detected in negative and positive modes. In negative mode, only one ion was detected for each by-product: 333 uma for A and 345 uma for B which would correspond to masses of 334 and 346 g mol^{-1}, respectively. In positive mode, the spectra were more complex because three ions were detected for each product (see Table 4). A similar pattern was observed beside the central ion (i.e. 335 uma (A) and 347 uma (B)). Lower mass ions were detected at 317 uma (A) and 329 uma (B) corresponding to a difference of −18 with respect to central ions. Higher mass ions were detected at 349 uma (A) and 361 uma (B) corresponding to a difference of +14

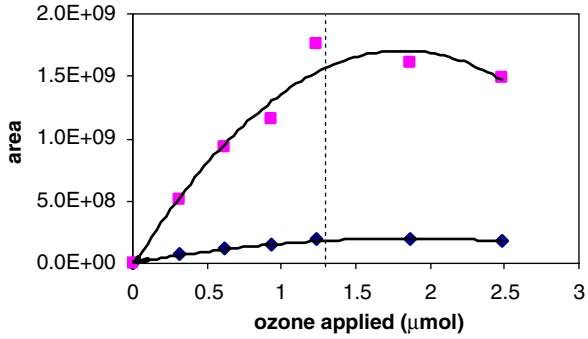


Fig. 7 – Area evolution of A and B by-products as a function of ozone dose applied: ◆ by-product A; ■ by-product B; - - - stoichiometric reaction (i.e. 100% of progesterone conversion).

with respect to central ions. According to these observations and according to the results obtained in negative mode, we supposed that the masses of products A and B were 334 g mol^{-1} and 346 g mol^{-1}, respectively. In positive ionisation mode, water elimination in the ionisation source would lead to 317 uma (A) and 329 uma (B) ions and a further solvent addition (+32 i.e. methanol) would lead to the formation of 349 uma (A) and 361 uma (B) ions.

- MS^2 experiments
  To get a better description of by-products' structures, MS^2 experiments were performed in the ion trap. Information is gathered in Table 4:

Progesterone: The isolation and fragmentation of the molecular peak at 313 uma in negative APCI led to the formation of two fragments at 285 and 295 uma, respectively consistent with CO and H_2O eliminations.
Compound A: The isolation and fragmentation of the main peak obtained in negative APCI (333 uma) led to two peaks of 289 and 261 uma (spectra not shown). This evidenced a loss of CO_2 and a probable loss of CH_2CH_2COOH according to progesterone structure.
Compound B: The isolation and fragmentation of the main peak obtained in negative APCI (345 uma) led to two peaks of 317 and 261 uma (spectra not shown). This evidenced a

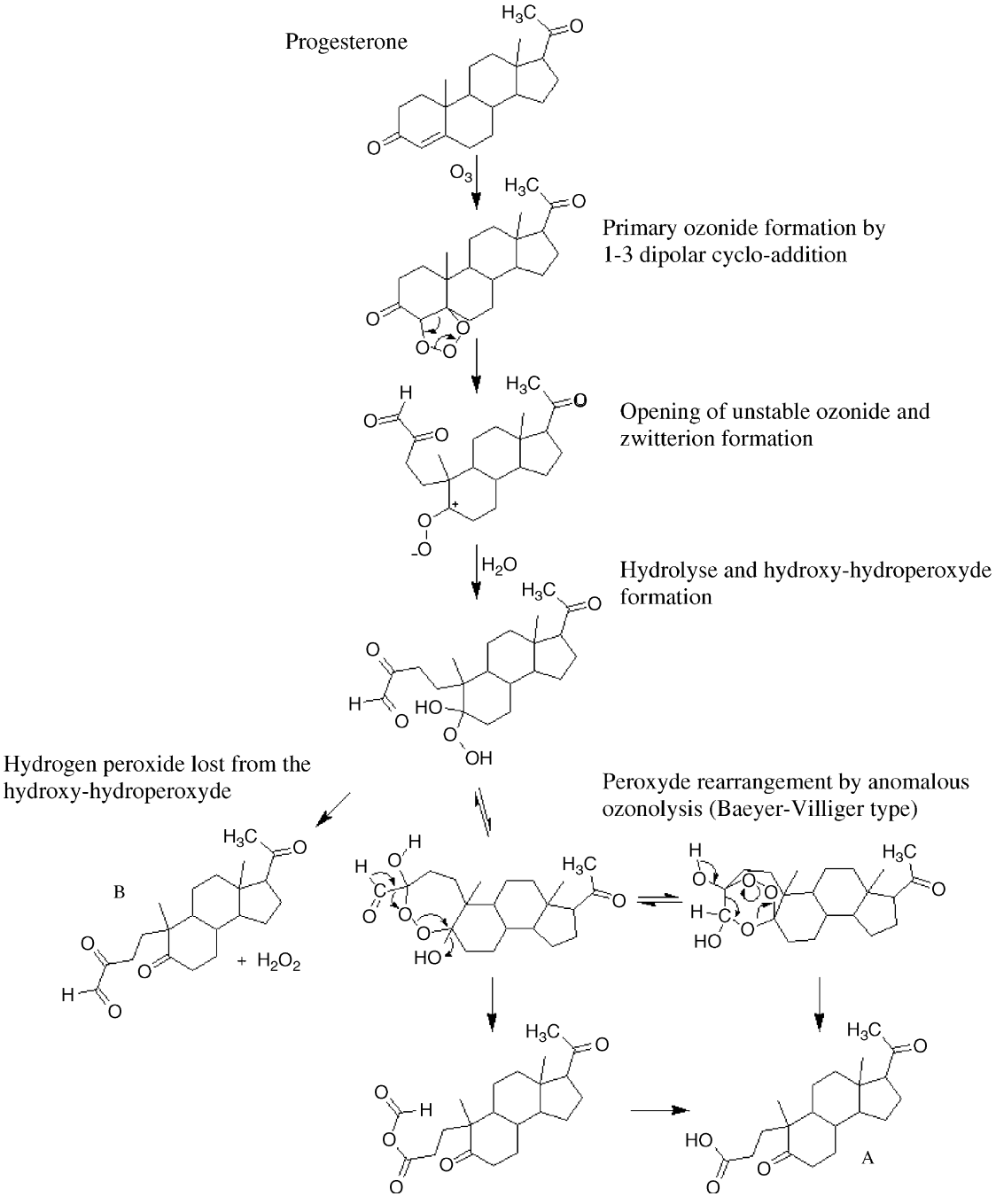


Fig. 8 – Proposed reaction pathway for the formation of A and B products.

loss of CO and a probable loss of CH_2CH_2COCOH. It was worth noting the formation of the same fragment at 261 uma for both by-products.

MS^2 experiments of molecular peaks obtained in positive APCI mode confirmed the hypothesis of adduct formation with methanol but did not give additional structural information. It could also be noted that MS^2 experiments performed in positive ionisation mode only showed water elimination certainly arising from the ceto-enolic equilibrium.

According to progesterone structure, information obtained by MS^2 experiments and our knowledge of ozone way of reaction, we could put forward the two structures represented in Fig. 6 for A and B by-products.

The reactional pathway for the formation of products A and B is proposed in Fig. 8. First, ozonide could be formed by ozone cyclo-addition on unsaturated bond (Bailey, 1978; Legube, 2003; Von Gunten, 2003). The cleavage of the unstable ozonide led to the formation of zwitterionic species. Then, the hydroxy-hydroperoxyde could develop the two by-products A and B. A could be formed by an anomalous ozonolysis mechanism inspired from Baeyer–Villiger mechanism. This latter mechanism was similar to the one described by Dauben et al. and Lefebvre et al. for cholestenone as reported by Bailey (1978). By-product B formation could be explained by hydrogen peroxide liberation from the hydroxy-hydroperoxyde form. Hydrogen peroxide formation was confirmed by titanium chloride method. Actually, a continuous formation of H_2O_2 was observed during ozonation of progesterone ($[P]_0 = 15.2\,\mu M$). Hence, for 51% and 98% of degraded progesterone, H_2O_2 concentrations determined were respectively 2.7 and 6.0 μM. This experiment seemed to confirm the liberation of H_2O_2 according to path B.

Additional experiments were realised with excesses of ozone (1.5–2 moles/mole) in presence of tert-butanol (10 mM). Fig. 7 presents an example of results where peak integration (area in negative mode) was reported versus ozone dose. It showed that A and B accumulated in the solution even in the presence of ozone in excess. This observation was in agreement with the structure of A and B in which no ozone reactive site could be clearly identified (Fig. 8).

4. Conclusions

This work dealt with kinetic and mechanistic points of view of progesterone ozonation reaction. Kinetic experiments with tert-butanol allowed rate constant estimation of $480 \pm 30\,M^{-1}\,s^{-1}$ for pH ranging between 2.0 and 8.0 (temperature $18 \pm 1\,^{\circ}C$).

Mechanistic studies showed that reaction stoichiometry was about one and allowed the identification of two major by-products. These compounds came from progesterone ozonide formation, hydration and either loss of hydrogen peroxide molecule or Baeyer–Villiger rearrangement type.

The biological effects of by-products need to be assessed to have a complete description of progesterone elimination by ozone.

In conditions closed of those applied in drinking water process (ozone concentration about 0.4–1 mg L^{-1}), progesterone calculated half-life time is between 1 and 3 min (this estimation does not take into account radical reactions). In those conditions, progesterone is expected to be significantly removed by station using ozone contrary to station using chlorine.

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