Progestagens for human use. Exposure and hazard assessment for the aquatic environment

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Capsule:
Gestagens exposure and hazard assessment for the aquatic environment.

Keywords:
gestagens; metabolites; surface water; hazard; exposure; assessment

Abstract:

Little information is available on the environmental occurrence and ecotoxicological effects of pharmaceutical gestagens released in the aquatic environment. Since eighteen different gestagens were found to be used in France, preliminary exposure and hazard assessment were done. Predicted environmental concentrations (PECs) suggest that if parent gestagens are expected to be found in the $ng.l^{-1}$ range, some active metabolites could be present at higher concentrations, although limited data on metabolism and environmental fate limit the relevance of PECs. The biological effects are not expected to be restricted to progestagenic activity. Both anti-androgenic activity (mainly for cyproterone acetate, chlormadinone acetate and their metabolites) and estrogenic activity (mainly for reduced metabolites of levonorgestrel and norethisterone) should also occur. All these molecules are likely to have a cumulative effect among themselves or with other xenoestrogens. Studies on occurrence, toxicity and degradation time are therefore needed for several of these compounds.

1. Introduction:

It is now recognized that pharmaceutical compounds reach the aquatic environment. A wide range of drugs (antibiotics, antidepressants, nonsteroidal anti-inflammatories, blood lipid-lowering agents, anti-hypertensors and so on) have been found in wastewater treatment plant (WWTP) effluents and surface waters, at concentrations ranging from the $ng.l^{-1}$ to the $\mu g.l^{-1}$ (Halling-Sorensen et al., 1998; Ternes, 1998 ; Kolpin et al., 2002 ; Kümmerer, 2004). Therefore, more and more studies are directed toward assessing the risk of human pharmaceuticals in the aquatic environment. In this context, the question of the potential risk of endocrine disruption due to hormones used in contraception and hormone replacement therapy (HRT) needs to be addressed. It is well known now that many chemicals capable of endocrine disruption are found in the aquatic environment (Colborn et al., 1993; Sumpter, 2005), but the contribution of human pharmaceuticals to this contamination has not yet been defined.

A large number of studies investigating the occurrence and effects of natural and synthetic estrogen steroids (ethinylestradiol, estradiol, estrone and estriol) and estrogen-like molecules have been conducted, and the risk is now well documented. A few studies have been conducted on the risk related to anti-androgens (Sumpter, 2005), and surprisingly, virtually no studies have been conducted on the occurrence of gestagens in wastewater and surface water and their effect on non-target organisms.

Gestagens (also called progestogens, progestagens or progestins) are hormones that produce effects similar to those of progesterone (P4). Progesterone is a C-21 steroid hormone involved in the female menstrual cycle, pregnancy and the embryogenesis of humans and other species (Rozenbaum, 2001; Hardman et al., 1996). Since natural progesterone is

inactivated very rapidly in the organism, several synthetic progestins have been developed. Progesterone and synthetic progestins act through nuclear receptors, mainly the progesterone receptor (PR) but also through other receptors such as the androgen receptor (AR), estrogen receptor (ER), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). Synthetic progestins can have various hormonal activities: estrogenic, anti-androgenic and androgenic (Table 1).

Gestagens are compounds that can pose a risk for the aquatic environment, at least for fish, in which gestagens play a role in the control of spawning behavior (Kobayashi et al., 2002). In a recent study (Kolodziej et al., 2003), the synthetic progestin medroxyprogesterone acetate (MPA) and other steroid hormones were found in WWTP effluent samples. The authors highlighted that, considering the levels found, these compounds were able to elicit pheromonal responses in fish that could alter their behavior and interfere with their reproduction (Kolodziej et al., 2003).

Reviewing the use of steroid hormones in France, we found that there were 18 different natural and synthetic gestagens used but only few occurrence studies and no environmental risk assessments. In this paper we therefore propose to review the knowledge on progestins and to conduct a preliminary assessment of the risk for wastewaters and surface waters.

2. Pharmacology of gestagens:

2.1 Classification of gestagens:

Progesterone is the only natural gestagen: all other molecules are synthetic and are often referred to as progestins or synthetic progestins. In this paper, the terms “gestagen” and

"progestin" will be used throughout to describe gestagens in general and synthetic progestins, respectively. Synthetic progestins differ from progesterone by several chemical modifications (Appendix A) and are classified accordingly. Briefly, there are derivatives of 17-α-hydroxyprogesterone (pregnanes), of 17-α-norhydroxyprogesterone and 19-norprogesterone (norpreganes), and of 19-nortestosterone (estranes and gonanes). There are large differences in the biological effects of progestins: some can present estrogenic activity (norethisterone, tibolone) while others are well known to display anti-androgenic activities (cyproterone and chlormadinone acetate); these different properties are summarized in Table 1.

2.2 Mechanism of action and activity of gestagens in mammals:

Natural progesterone (P4) has progestagenic, anti-estrogenic, anti-aldosterone and mild anti-androgenic activities. P4 mainly acts through the progesterone nuclear receptors PRA and PRB and modifies the transcription of target genes (Rozenbaum, 2001). The main genomic activities are:

- preparation of the uterus for nidation, then maintaining gestation.
- inhibition of the secretion of pituitary gonadostimulins,
- blastocyte implantation,
- anti-estrogenic effect by induction of 17-hydroxysteroid dehydrogenase, which accelerates the conversion of estradiol into estrone, and by induction of estrogen sulfotransferase.
- anti-mineralocorticoid action by inhibition of aldosterone receptors, which induces a decrease in plasma sodium by increasing its urinary elimination.

P4 can also produce non-genomic effects. Such effects are reported to be faster than genomic effects (hours versus days) and can trigger different metabolic reactions and receptors (GABA, NMDA and acetylcholine receptors). P4 plays a role in oocyte maturation and modulation of reproductive signaling in the brain, but also has a sedating action by potentiating the GABA effect on GABA(A) receptors or can impair the glucose metabolism (Hardman et al., 1996; Rozenbaum, 2001; Pharmacorama, 2008).

Synthetic progestins are mainly used in association with an estrogen in oral contraception. Progestin activity may differ from natural P4 (Table 1): some can display estrogenic activities (tibolone [TBL], levonorgestrel [LNG]), while others are stronger anti-androgenics (cyproterone acetate [CPA]). Synthetic progestins are generally more potent than P4; as an example they are reported to be much more effective inhibitors of the secretion of gonadostimulins (Pharmacorama, 2008). Progestins also have several other metabolic effects, depending on their chemical structure. For example, ethinylated gestagens, particularly gestodene (GSD), have been demonstrated to inhibit cytochrome P450 enzymes (Rozenbaum, 2001; Kuhl, 1996).

2.3.Structure activity relationships:

As noted above, progestins can have subtle differences in their mode of action. This is related to chemical modifications at key points of the general structure of gestagens (Rozenbaum, 2001; Stanczyk, 1996). By acting on these key points, it is possible not only to modulate the progestagenic activity but also to switch the activity to other steroid pathways. The key points are shown in Figure 1 and the structure–activity relationships are summarized in Table 2.

2.Material and methods:

This paper has two main goals: i) to review the ecotoxicological and pharmacological knowledge on progestogens used in human medicine to provide an overview of the biological effects of these molecules and ii) to assess the exposure to the aquatic environment by calculating predicted environmental concentrations (PECs) and to provide a preliminary characterization of the risk for gestagens.

To do so, the scientific literature was reviewed, as well as the following databases and books: the Banque Claude Bernard (BCB), a complete, free French databank on human pharmaceuticals (http://www.resip.fr), the BIAM database (www.biam2.org), the drugs.com drug database (www.drugs.com), the Micromedex Drugdex® databank (from Thomson Micromedex, available at www.micromedex.com/products/drugdex), the Martindale compendium's *Complete Drug Reference* (Martindale 2002), *Les progestatifs* (Rozenbaum, 2001) and the Goodman and Gilman's *The Pharmacological Basis of Therapeutics* (Hardman et al. 1996). Special attention was paid to the metabolism and active metabolites of gestagens. As highlighted in previous studies on pharmaceuticals, metabolism is one of the most important processes that can reduce the quantities of pharmaceuticals reaching the aquatic environment. Moreover, human metabolism can give rise to metabolites (Figure 2) that must be considered when assessing the environmental risk (Besse et al., 2008; Huschek et al., 2003). Finally, as highlighted above in the structure–activity relationships, modifications in chemical structure can result in important modifications in activity, both quantitatively and qualitatively.

Preliminary exposure assessment was implemented by calculating PECs, as described in Besse et al. (2008), using the following equation:

$$PECsurfacewater = \frac{consumption \times Fexcreta \times Fstp}{WWinhab \times hab \times Dilution \times 365}$$

PEC is expressed in $mg.l^{-1}$ using the following parameters: consumption is the quantity ($mg.year^{-1}$) of an active molecule consumed by the population over 1 year in a defined zone (generally a country); hab is the number of inhabitants and 100 the correction factor for the percentages; 365 is the number of days per year ($day.year^{-1}$); WWinhab is the volume of wastewater per person per day (default value = 200 l. $inhabitants^{-1}$ day^{-1}); dilution is the dilution factor from WWTP effluent to surface waters (default value set at 10); Fexcreta is the excretion fraction of the active molecule; Fstp is the fraction of emission of the drug from wastewater treatment plants (WWTPs) directed to surface water, which can be defined as (1-WWTP removal fraction).

PEC gestagens were calculated using the actual amounts of progestogens provided by the French Medical Product Safety Agency (Agence Française de Sécurité Sanitaire des Produits de Santé, AFSSAPS, Paris).

3. Results

3.1.Metabolism data

Metabolism and pharmacokinetic data for progestins are limited (Stanczyk, 2003). No reliable excretion fraction value could be determined, except for cyproterone acetate (CPA). However, reviewing metabolism data highlighted very valuable information on the metabolites of gestagens, particularly progestins, that can help describe the hazard for the aquatic

environment. All gestagens are metabolized extensively following the same general pathway: in the liver, they are mainly submitted to reduction and hydroxylation. Reduction generally acts primarily on a double-bond of ring A then on the ketone function of carbon 3. These structural modifications lead to metabolites that can be pharmacologically active but whose activity differs from the parent compounds. The parent compound and metabolites can subsequently be sulfo- and glucuroconjugated prior to excretion (Stanczyk, 2003; Rozenbaum, 2001). Information on gestagen metabolites is summarized in Table 3 and detailed in Appendix B. Although some metabolites with progestagenic properties are formed, it should be noted that some metabolites have a significant *in vitro* estrogenic activity (Garcia-Becerra et al., 2002; Larrea et al., 2001). This has been shown for metabolites of gestagens structurally related to testosterone (Appendix B) and has a strong implication for the hazard and risk assessment related to gestagens. For medrogestone, nomegestrol acetate, promegestone, norgestrienone and norelgestromin, no data were found.

3.2. PEC calculation and comparison with field measurements:

Since no quantitative excretion data were available other than for CPA, the calculation of PEC values for progestogens remains limited and only conservative PEC values, assuming no metabolism, could be calculated. Moreover, since very few studies have been conducted on progestagens in the environment, no data exist on the WWTP removal rates for these molecules. Therefore, to limit the uncertainties, we calculated PEC values for the WWTP influent, without considering the WWTP removal rate and dilution factor. The results are displayed in Table 4. Since lynestrenol and norgestimate are prodrugs, their consumption amounts were summed with the amounts of norethisterone (NET) and levonorgestrel (LNG),

their corresponding active metabolite, respectively, to calculate a more accurate PEC. PEC values for progestins range from less than the ng.l^{-1} to the hundred ng.l^{-1} level. Norelgestromin and norgestrienone show very low PEC values, even with conservative assumptions; consequently, these two molecules are not expected to be present in the aquatic environment. P4 showed a high PEC of more than 2 µg/l; however, like all other gestagens, P4 is submitted to extensive metabolism and only traces of the parent compound are excreted; therefore, lower concentrations are expected in the aquatic environment. Moreover, it has been shown that P4 was highly removed in WWTPs, with a high proportion sorbed on sludge. (Esperanza et al., 2007).

Only a few studies on the occurrence of progestagens in the environment have been conducted to date and LNG and NET are the main progestagens that have been investigated. Kuch and Ballschmitter (2000) did not find NET acetate in WWTP effluents, whereas LNG was found in only one sample, at the low concentration of 1 ng.l^{-1}. A study in French rivers did not detect LNG or NET, (Labadie and Budzinski, 2005a). Another study (Solé et al., 2000) did not reveal the presence of LNG, NET or progesterone in surface waters and WWTP effluents. Other studies (Petrovic et al., 2002; Lopez de alda et al., 2002) report the presence of NET, LNG and P4 in WWTP effluents or in sediment rivers (Table 5). Three very recent studies also report the occurrence of gestagens in different environmental samples (Table 5): P4 has been found in hospital and urban WWTP effluents in concentrations up to 33 ng.l^{-1} (Pauwels et al., 2008); several gestagens have been measured (Chang et al., 2008) in the low ng.l^{-1} range in effluents and surface waters; P4, LNG and NET have been found in surface water and groundwater samples at the ng.l^{-1} range (Vulliet et al., 2008). Finally, medroxyprogesterone acetate (MPA) was found in effluent samples (Kolodziej et al., 2003). MPA was detected in four of eight effluents tested, at concentrations ranging from 1 ng.l^{-1} to

15 ng.l^{-1}. MPA was also detected at low concentrations (0.4–0.7 ng.l^{-1}) in wetland samples. For MPA, the maximum effluent value of 15 ng.l^{-1} found in one effluent sample (Kolodziej et al., 2003) is equal to the conservative PECs determined for influents, suggesting that this molecule could be excreted in significant amounts. Since PECs remain conservative because of limited data, the calculated values are much higher than the range of the measured values (i.e., ng.l^{-1}). This difference could be mainly related to extensive metabolism and low excretion amounts of the unchanged parent molecule.

4. Discussion:

4.1. Concentrations entering the aquatic environment

Given the limited metabolism data, most particularly the lack of quantitative excretion data, it was only possible to calculate conservative PECs, which limits their environmental relevance. Although it was not possible to calculate the excretion fraction for gestagens, some evidence (Verhoeven, 2001; Stanczyk, 1990; Vos, 2003) suggest that natural progesterone and some progestins related to testosterone (i.e., LNG, NET, ETO and GTD) have excretion fractions of a free and/or conjugate unchanged molecule ranging from about 5 to 10%. Applying this excretion fraction range to calculated PECs gives refined values in the range of field measurements. Field measurements from the study of Kolodziej et al. (2003) suggest that MPA could be excreted in higher amounts as an unchanged molecule.

Some gestagen metabolites could be present at higher concentrations. Although no excretion value could be calculated, there is some evidence in the literature for higher excretion rates than parent compounds.

Concentrations of pregnanediol (main metabolite of P4) entering the aquatic environment could be of environmental concern. Assuming a 15% excretion fraction value for this compound (Grady et al., 1952; Sommerville and Marrian, 1950) gives a PEC for WWTP influent of about 340 $ng.l^{-1}$. Pregnanediol is also physiologically excreted in urine, in daily rates ranging from 1 mg for men to 70 mg for pregnant women (Hardman et al., 1996), giving a cumulated excretion over a year of about 150 kg for France (data not shown). Added to the assumed PEC levels described above, this gives concentrations entering WWTPs of about 370 $ng.l^{-1}$. This metabolite is reported to be inactive. Moreover, it has undoubtedly been present in the environment for decades, and there is no evidence that any biological effect is related to it. However, considering the rates of its release into the environment through WWTPs, which should have considerably increased with the use of P4 in HRT, concerns are being raised and studies should investigate the fate and toxicity of this molecule.

Other metabolites with estrogenic activity could be found in surface waters at higher levels than parent compounds. Following an oral administration of NET (Stanczyk and Roy, 1990), there was five times more 3α-5β-tetrahydro-NET (free and conjugated) than NET (free and conjugated) in urine. The same study showed that total urinary amounts of 3α-5β-tetrahydro-LNG (free and conjugated) were 15 times higher than total amounts of LNG. Although there is a need for more studies, especially to determine WWTP removal rates of these molecules, these results indicate that wastewater effluent levels for 3α-5β-tetrahydro-NET and 3α-5β-tetrahydro-LNG could be up to 45 $ng.l^{-1}$ and 240 $ng.l^{-1}$, respectively (by taking into account maximum values for NET and LNG reported in Table 5 and assuming comparable removal rates for parent compounds and metabolites). Therefore, there is evidence that most relevant compounds according to the environmental exposure and biological activity have not been targeted yet.

295

296 *4.2. Fate and behavior in the aquatic environment*

297

298 Except for P4, for which high removal rates in WWTPs and rapid degradation in surface
299 water are expected (Esperanza et al., 2007; Labadie and Budzinski, 2005b), no data are
300 available on the environmental half-lives and degradation pathways for gestagens. This is a
301 significant limitation for the environmental assessment of gestagens and limits the relevance
302 of the environmental concentrations of metabolites calculated above. It is not possible to draw
303 any conclusion on the degradability of these molecules, but the following assumptions can be
304 made. First, the degradability of synthetic progestins could be lower than for natural P4,
305 although this is an indirect assumption. Synthetic progestins are specifically designed to have
306 higher body half-lives than P4, which is rapidly inactivated. Moreover, a parallel can be
307 drawn between estrogens and gestagens. Synthetic ethinylestradiol (EE2) is reported to be
308 more resistant to bacterial biodegradation than natural estradiol (E2) (Ying et al., 2003;
309 Jürgens et al., 2002), whereas the photodegradation times are similar (Jürgens et al., 2002).
310 Resistance to bacterial degradation could be related to differences in stereochemistry and the
311 presence of a C17 ethinylated group in EE2. Similarly, synthetic progestins (and their
312 metabolites) could be more resistant to bacterial biodegradation, especially NET, LNG, TBL
313 and norgestimate, which also have an ethinylated function on C17 (Appendix A) but also
314 CPA, MPA and CMA, which display acetate groups on C17 (Appendix A). Second,
315 pharmaceuticals are often considered to be pseudo-persistent contaminants (Daughton, 2003),
316 because they are introduced in the aquatic environment on a continual basis through WWTPs.
317 Given that gestagens are used throughout the year for oral contraception and HRT, this
318 continuous release could balance a possible rapid degradation.

Concentrations of gestagens in sediment could be higher than those found in the water column. This is suggested by the results of a study (Jenkins et al., 2003) in which P4 levels found in sediment were 20 times higher than those found in the water column. Moreover, Kd values for NET and P4 have been calculated (Lopez de alda et al., 2002) and the results indicate that gestagens may have a general tendency to accumulate in sediments, but this remains to be confirmed.

4.3. Biological effects on aquatic species

Several progesterone receptors have been found in fish (Pinter and Thomas, 1995; Todo et al., 2000; Zhu et al., 2003) and gestagens play major roles in fish reproduction. They trigger oocyte maturation (Lutes, 1985; Truscott et al., 1992) and ovulation in female fish (Scott et al., 1983; Pinter and Thomas, 1999; Venkatesh et al., 1991). They also play a role in the spermiation in males (Barry et al., 1990). Therefore, there is evidence that synthetic progestins could interfere with endogen gestagens in fish and adversely affect their reproduction. Moreover gestagens also act as pheromones in fish and are involved in the control of spawning behavior (Kobayashi et al., 2002). At environmental concentrations, gestagens could interfere with natural pheromones and therefore impair the physiological responses and spawning behavior in fish (Kolodziej et al., 2003; Sorensen et al., 1990). However, except for CPA, which was assessed for its anti-androgenic properties, no ecotoxicological data in fish were found.

Very few data are available on the ecotoxicity of gestagens on aquatic invertebrates. A very recent study, assessing the effects of estradiol, EE2 and MPA on the cladoceran *Ceriodaphnia dubia*, showed no effect on reproduction at concentrations ranging from 5 $\mu g.l^{-1}$ to 5 $mg.l^{-1}$

(Jukowski et al., 2008). On the contrary, P4 was shown to induce the production of more male-dominated broods in a 25-day assay at the concentration of 100 $\mu g.l^{-1}$ (Kashian and Dodson, 2004). Another study (Goto and Hiromi, 2003) determined a 48-h EC_{50} for NET on immobilization on *Daphnia magna* of 6.41 $mg.l^{-1}$, which is very higher than environmental concentrations. No chronic toxicity was observed for NET on the total number of offspring, reproduction frequency, total number of male offspring, and total number of molts at tested concentrations (< 500 $\mu g.l^{-1}$), but the results suggested that there was a synergistic effect between EE2 and NET and the tested parameters.

No ecotoxicological data are available for metabolites of progestins; however, pharmacological data i) show that some of these metabolites have a pharmacological activity and ii) suggest that they could be found at higher concentrations than parent compounds in the environment. Therefore, there is a potential for biological effects on non-target organisms. The risk could be linked to mixture of gestagens. Gestagens used by humans are only found at very low concentrations in aquatic samples; however, a number of different molecules (18 in France) are used. Moreover, since parent molecules show a main similar mode of action, there is a real risk of additive or synergistic effects in the environment. Finally, there is a risk of synergistic effects between gestagens and estrogens, as shown for P4 and EE2 (Goto and Hiromi, 2003) and as such effects have been shown in humans (Rozenbaum, 2001).

4.4. Hazard characterization:

Although ecotoxicological data are still too limited to provide information for the hazard assessment of gestagens, the review of pharmacological data can give valuable hints on the biological effects of gestagens and their metabolites. Surprisingly, the potential hazard posed

by these molecules may not be limited only to the progestagenic activity, but also to estrogenic and to anti-androgenic activity. The different types of biological activities to the different molecules are discussed here and are summarized in Figure 3. The hazard characterization provided here is limited to aquatic species that display similar receptors to those found in mammals; for other species, other effects (if any) should occur.

Progestagenic activity. The risk of progestagenic activity is obviously the first to consider. Natural progesterone and parent progestins have a significant binding affinity and transcriptional activity on the PR. Synthetic progestins have a higher activity than natural progesterone. Dihydro metabolites of progestins have substantially less binding affinity to PR than the parent compound but within the same range as progesterone. This has been shown *in vitro* for GES, NES and LNG (Larrea et al., 2001; Lemus et al., 2000; García-Becerra et al., 2002). Some metabolites that can be excreted in higher amounts than parent compounds also have a significant activity: DHD, 17-hydroxyprogesterone and 20α-dihydroprogesterone, Δ4-tibolone, and to a lesser extent, reduced metabolites. Therefore, there is a potential hazard of progestagenic activity in the environment. Considering the low measured concentrations in the environment, the risk posed by progestagenic activity could be mainly related to the mixture of gestagens and their metabolites.

A very recent study (Van der Linden et al., 2008) used several modified CALUX assays to detect hormonal activities in water samples. These authors showed that there were multiple hormonal activities in effluent samples. Progestagenic activity was found in only half of the samples, whereas estrogenic activity and glucocorticoid activity was found in all the samples. Higher progestagenic activity was found in an industrial effluent sample than in municipal effluents, suggesting that the contribution of gestagens used by humans to the aquatic

391 contamination is not the major one. No activity was found in paper mill effluent, whereas a
392 previous study (Jenkins et al., 2003) detected natural progesterone in sediment and surface
393 waters exposed to paper mill effluent in concentrations 100 times higher than at a reference
394 site. No progestagenic activity was found in hospital effluents, perhaps because most of the
395 progestagen consumption is used for hormone replacement therapy or contraceptives and is
396 therefore used outside hospitals. Nevertheless, P4 has been found in hospital WWTPs
397 (Pauwels et al., 2008).
398 PR activity was only found in a small stream but not in river waters (Van der Linden et al.,
399 2008). This result suggests the absence or at least very low concentrations of gestagens in
400 surface waters. There is a need for more studies since this result may stem from several
401 factors: i) the low half-life for gestagens, as has been shown for P4 (Labadie and Budzinski,
402 2005b); ii) the sorption of gestagens in sediment or suspended matter (Lopez de alda et al.,
403 2002; Esperanza et al., 2007); and iii), a lack of sensitivity of the ER CALUX assay. The
404 reference compound used was org2058, reported to be 13 times more potent than
405 progesterone in this assay. Given that most gestagen metabolites have a progestagenic activity
406 similar to or lower than progesterone (Appendix B), it is possible that the use of org2058 as
407 the reference compound is not sensitive enough to detect an environmental progestagenic
408 activity.
409
410 *Estrogenic activity.* Progestins derived from testosterone appear to transform into
411 tetrahydroderivatives, which present an estrogenic activity. This had been shown *in vitro* for
412 GSD (Larrea et al., 2001; Lemus et al., 2000), NET (Larrea et al., 2001) and LNG, although
413 there are some contradictory results on this last molecule (Garcia-Becerra et al., 2002; Lemus
414 et al., 1992). The 3β-5α-tetrahydroderivatives from NET, GES and LNG have been shown to

bind with the α subunit of the ER and displayed *in vitro* transcriptional activities through the Erα equivalent to about 90% of that of estradiol for tetrahydro-NET and tetrahydro-GES (Larrea et al., 2001) and equivalent to about 80% for tetrahydro-LNG. Such derivatives are structurally close to ethinylestradiol (Appendix A). Dihydroderivatives also display, but to a lesser extent, an estrogenic activity through ERα (Larrea et al., 2001; Garcia-Becerra et al., 2002). Moreover, TBL is metabolized into two main metabolites that are known to exhibit estrogenic activity: 3α and 3β-hydroy-TBL (Schindler et al., 2003, Rozenbaum, 2001). Therefore, there is evidence that some gestagen metabolites could act as estrogenic compounds in the aquatic environment. The environmental risk of estrogenic disruption remains putative as *in vivo* human tetrahydro-isomers (3α-5β, major isomer) are different from those that have been tested *in vitro* (3β-5α form). Since there can be differences in the estrogenic activity of isomers (Larrea et al., 2001; Lemus et al., 2000), there is a need to clearly assess the estrogenic activity of 3α-5β-tetrahydroderivatives. Nevertheless, since NES and GES are reported to have estrogenic activity (Table 1), there is strong evidence that their metabolites display this activity.

As discussed above, concentrations of these metabolites entering the aquatic environment could be higher than those of parent compounds; therefore, there is a potential risk of estrogenic activity. They have a similar mode of action to estrogen: consequently, tetrahydroderivatives and to a lesser extent, dihydroderivative metabolites from progestins derived from testosterone are likely to contribute to an estrogenic risk for the aquatic environment, by cumulative effect with each other and/or with other natural or xenoestrogens such as ethinylestradiol.

Anti-androgenic activity. A risk of anti-androgenic activity cannot be excluded. CPA, CMA and DSP are progestins that have an anti-androgenic activity. Hydroxylated metabolites of CPA and CMA are reported to be anti-androgenic as well, so there is a possibility that the mixture of these molecules is present in the aquatic environment in sufficiently high concentrations to elicit anti-androgenic responses in non-target organisms. Exposure of snails to nominal concentrations of 1.25 mg/l of CPA resulted in reduced length of male sex organs (Tillmann et al., 2001); however, the authors concluded that anti-androgens could be of lesser concern for snails compared with estrogens and androgens. The results of a study on exposure of Japanese medaka to anti-androgens (Kiparissis et al., 2001) concluded that CPA had the potential to alter testicular development and gametogenesis in fish. However, tested concentrations were in the $\mu g.l^{-1}$ range, higher than what is expected in the aquatic environment for CPA. In a 7-day experiment, exposure of fish to 250 $ng.l^{-1}$ CPA resulted in decreasing circulating levels of estradiol and testosterone (Sharpe et al., 2003). In the same study, a significant decrease in plasma testosterone levels in males exposed to 100 $ng.l^{-1}$ and 10 $ng.l^{-1}$ in females was observed after 14 days. Vitellogenin plasma levels were not affected by CPA exposure at the tested concentrations. Since the excretion fraction of unchanged CPA are reported to range from 5 to 20% (Appendix B), the influent PEC ranges from 10 to 40 $ng.l^{-1}$, a comparable concentration that elicited effects in fish. Concentrations of CPA in surface waters could be lower, depending on WWTP removal rates and degradation time. On the other hand, other anti-androgenic compounds such as CMA and hydroxylated metabolites of CMA and CPA could act additively. There is a potential risk of anti-androgenic activity related to gestagens, although there is a need for more studies.

4.5. Selection of relevant gestagens according to the environmental concern

462

463 In previous work on pharmaceuticals (Besse and Garric 2008), a prioritization was conducted
464 on in order to identify the compounds of high concern for the environment. The prioritization
465 strategy previously implemented was not applicable to gestagens mainly because of the lack
466 of a reliable excretion fraction. Theoretically, it could be possible to rank gestagens and their
467 metabolites by comparing their consumption amounts and their relative binding affinities to
468 PR and ER, but even in this case, the available data are too limited and heterogeneous to
469 classify them accurately.
470 Nevertheless, it is possible to target a few compounds for which occurrence studies and
471 ecotoxicological assays should be implemented firstly, to assess the risk of endocrine
472 disruption, in accordance with each respective type of risk (i.e., progestagenic, estrogenic and
473 anti-androgenic). This risk of endocrine disruption for aquatic species is based on the
474 biological activity observed in mammals, therefore, it is limited to fishes and possibly to
475 invertebrates that display similar steroid receptors than in mammals.
476 MPA is the parent progestin that has been found in the highest amounts (Kolodziej et al.,
477 2003); therefore, other occurrence and toxicity studies should be directed toward MPA.
478 Occurrence studies should also be conducted on DHG, which is the third-ranked molecule in
479 terms of consumption amounts (Table 5). As DHG is mainly metabolized in the active DHD,
480 occurrence studies should also investigate this last molecule.
481 Tetrahydro-metabolites of LNG, GSD and NET, and the metabolites of TBL, are likely to
482 exert an estrogenic activity. Since GSD is used in low amounts (Table 5), concentrations of
483 this compound and its metabolites entering the environment are expected to be negligible. On
484 the contrary, occurrence and ecotoxicological studies should focus on 3α,5β tetrahydro-
485 metabolites of LNG and NET and on 3α hydroxylated metabolite of TBL.

CPA, CMA and their hydroxy metabolites should be tested for occurrence and toxicity because they display a significant anti-androgenic activity.

Given that pregnanediol could be excreted in significant amounts, it should be tested for ecotoxicity and occurrence.

Finally, for all the molecules cited above, degradation studies are needed to determine their removal rates in WWTPs and their fate in the aquatic environment (sorption to sediment and degradation time).

Conclusion

This study was the first one conducted on the environmental risk of gestagens used by humans. Although it was not possible to conclude on the environmental risk due to limited data on the fate of these molecules, it was possible to assess the hazard and the biological effects of gestagens and their metabolites and also to target relevant metabolites for further studies. The following conclusions, which remain to be confirmed, can be drawn.

- Synthetic progestins are expected to be found in effluent samples and possibly in surface waters, mainly as metabolites, in concentrations possibly in the $ng.l^{-1}$ and even the 100 $ng.l^{-1}$ range.
- Residual parent compounds and some active metabolites with progestagenic activity could interfere with spawning behavior in fish.
- Metabolites of progestins derived from nortestosterone can act as estrogenic compounds and therefore may act additively with other xenoestrogens such as ethinylestradiol.

- Some progestins such as chlormadinone acetate and cyproterone acetate and their metabolites have anti-androgenic properties and may pose a risk for aquatic species.
- Taken separately, progestins might not present a risk for the aquatic environment; however, since they are expected be found in the environment as mixtures, there is a risk of additive or even synergistic effects.

Therefore, as several other authors (Sumpter 2005; Johnson et al., 2008), we consider that synthetic gestagens merit more attention than they have received to date. There is a need to conduct other studies to clearly assess the risk for the aquatic environment, notably occurrence studies and ecotoxicological assays for metabolites and degradation tests for some parent gestagens and metabolites.

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