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Toxicity of prednisolone, dexamethasone and their photochemical derivatives on aquatic organisms

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Abstract

Light exposure of aqueous suspensions of prednisolone and dexamethasone causes their partial phototransformation. The photoproducts, isolated by chromatographic techniques, have been identified by spectroscopic means. Prednisolone, dexamethasone and their photoproducts have been tested to evaluate their acute and chronic toxic effects on some freshwater chain organisms. The rotifer *Brachionus calyciflorus* and the crustaceans *Thamnocephalus platyurus* and *Daphnia magna* were chosen to perform acute toxicity tests, while the alga *Pseudokircheneriella subcapitata* (formerly known as *Selenastrum capricornutum*) and the crustacean *Ceriodaphnia dubia* to perform chronic tests. The photochemical derivatives are more toxic than the parent compounds. Generally low acute toxicity was found. Chronic exposure to this class of pharmaceuticals caused inhibition of growth population on the freshwater crustacean *C. dubia* while the alga *P. subcapitata* seems to be less affected by the presence of these drugs.

Keywords: Prednisolone; Dexamethasone; Phototransformation; Acute and chronic toxicity

1. Introduction

The issue of drugs in the aquatic environment has raised increasing concern in recent years since these substances have the potential to impact both human and wildlife populations (Halling-Sorensen et al., 1998; Daughton and Ternes, 1999).

Many drugs, which are persistent, have been found in surface waters (Zuccato et al., 2000; Jones et al., 2002) and some toxicological studies on aquatic life organisms have revealed their harmfulness (Halling-Sorensen et al., 1998; Daughton and Ternes, 1999). Generally these investigations have reported only acute toxicity data, but it would be more relevant to perform also life cycle tests on organisms representing different trophic layers to identify the hazard of drugs in the aquatic environment. Furthermore, a little work has been devoted to the drugs that are not stable in the environment and are subjected to transformations. In such cases the analytical and toxicological assessments should be addressed towards their transformation products (Fig. 1).

In a recent study on prednisone (**1**) (DellaGreca et al., 2003) we have shown that this drug undergoes transformation by sunlight. After eight hours of exposure to a solar simulator, 60% of prednisone was transformed into seven photoproducts. The acute and chronic toxic effects of the parent compound and its photoderivatives were evaluated on a battery of different freshwater species and the results showed that some photoproducts were stable and more toxic than the parent drug.

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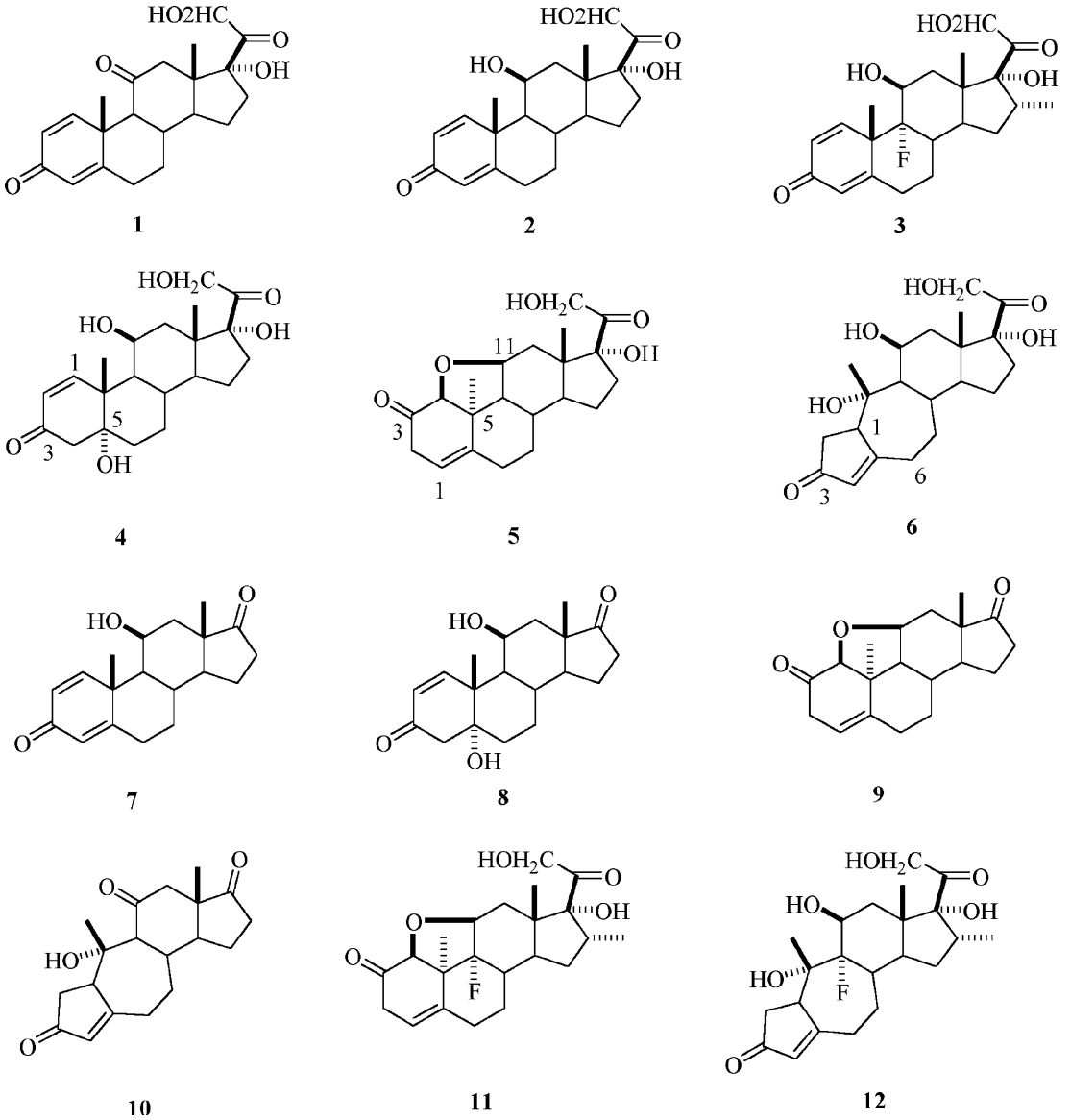


Fig. 1. Structure of compounds **1**–**12**.

Prednisone is a corticosteroid, a drug similar to the natural hormone cortisone used in several forms to treat many different conditions.

The wide range of pharmacological properties put corticosteroids among the most used drugs in the world, and in continuing our study on the fate and the toxicity of these substances we have now examined prednisolone (**2**) and dexamethasone (**3**). Both drugs are used for their potent anti-inflammatory effects and the first is a metabolite of prednisone in man (Maayan et al., 1988). Furthermore, both drugs are reported to be sensitive to light (Takacs et al., 1991).

The aim of the present study was to assess the behaviour of these drugs under sunlight irradiation and evaluate their acute toxic effects on primary consumers of the aquatic chain (the rotifer *Brachionus calyciflorus*, the crustaceans *Daphnia magna* and *Thamnocephalus platyurus*) as well as subtle effects through chronic tests on a producer (the alga *Pseudokircheneriella subcapitata*) and a consumer (the crustacean *Ceriodaphnia dubia*). Toxicity tests were performed on the parent pharmaceuticals and on their photoderivatives. Results demonstrate that phototransformation products can elicit severe chronic effects mainly on *C. dubia*.

2. Material and methods

2.1. General procedures

Nuclear magnetic resonance (NMR) spectra were recorded at 500 MHz for [^{1}H] and 125 MHz for [^{13}C] on a Fourier Transform NMR Varian 500 Unity Inova spectrometer and at 400 MHz for [^{1}H] and 100 MHz for [^{13}C] on a Bruker AC 400 spectrometer. The carbons multiplicity was evidenced by DEPT experiments. The proton couplings were evidenced by ^{1}H–^{1}H COSY experiments. The heteronuclear chemical shift correlations were determined by HMQC and HMBC pulse sequences. ^{1}H–^{1}H proximities through space within a molecule were determined by NOESY.

Electronic impact mass spectra (EI-MS) were obtained with a HP 6890 spectrometer equipped with a MS 5973 N detector. Infrared spectra (IR) were determined on a Fourier Transform Infrared Perkin-Elmer 1740 spectrometer in $CHCl_3$ solutions (0.025 M). Ultraviolet spectra (UV) were recorded in ethanol (10^{-4} M) on a Perkin-Elmer LAMBDA 7 spectrophotometer. High-performace liquid chromatography (HPLC) was performed on a Varian Vista 5500 apparatus equipped with a refractometric detector and Lichrosorb RP-8 columns. Irradiation experiments were performed with a 150 W solar simulator Oriel equipped with a Xenon lamp (spectral output 200–2400 nm).

2.2. Phototransformation of prednisolone (2)

A suspension of prednisolone (**2**) (100 mg) in water (500 ml) was irradiated by the solar simulator for 4 h under slow magnetic stirring. The reaction mixture was extracted with ethyl acetate and the residue was subjected to silica gel flash chromatography. Elution with $CHCl_3$–acetone (19:1) gave a mixture of products **7**–**10** (5 mg), while elution with $CHCl_3$–CH_3OH (19:1) gave unreacted prednisolone (**2**) (55 mg), pure **4** (14 mg) and crude **5** and **6**. TLC chromatography on silica gel ($CHCl_3$–CH_3OH 19:1) gave pure **5** (9 mg). Reverse-phase C-18 HPLC chromatography (H_2O–CH_3OH–CH_3CN 6:2:2) gave pure **6** (11 mg).

2.3. Synthesis of 1,4-androstadien-11β-olo-3,17-dione (7)

To a solution of prednisolone (**2**) (200 mg) in ethyl acetate (10 ml) MnO_2 (4 g) was added. After 1 h at room temperature the reaction mixture was filtered on celite eluting with ethyl acetate and methanol. Chromatography on silica gel ($CHCl_3$–acetone 19:1) of the filtrate gave 1,4-androstadien-11β-olo-3,17-dione (**7**) (170 mg).

2.4. Phototransformation of 1,4-androstadien-11β-olo-3,17-dione (7)

A suspension of 1,4-androstadien-11β-olo-3,17-dione (**7**) (180 mg) in water (1000 ml) was irradiated by the solar simulator for 4 h under magnetic stirring. The reaction mixture was separated by silica gel flash chromatography ($CHCl_3$–acetone 19:1) into its components **7** (140 mg), **8** (15 mg), **9** (8 mg) and **10** (20 mg).

2.5. Phototransformation of dexamethasone (3)

Dexamethasone (**3**) (100 mg) suspended in water (500 ml) was irradiated by a solar simulator for 8 h. The organic material was extracted with AcOEt (2×150 ml) and chromatographed by flash chromatography on silica gel. Elution with $CHCl_3$–acetone (7:3) gave three fractions A–C. Fraction A (85 mg) consisted of unreacted **3**. TLC chromatography on silica gel of fraction B ($CHCl_3$–CH_3OH 93:7) gave **12** (4 mg) while TLC chromatography (organic phase of the mixture hexane–CH_2Cl_2–CH_3OH–H_2O 10:40:17:8) of fraction C gave **11** (4 mg).

2.6. Chemicals

Prednisolone (**2**) and dexamethasone (**3**) were purchased from Sigma–Aldrich.

2.7. Acute toxicity testing

Acute toxicity was determined on primary consumers typical of the aquatic chain: the rotifer *B. calyciflorus* and the crustaceans *D. magna* and *T. platyurus*. All the organisms were provided in cryptobioticstages by MicroBioTests Inc., Nazareth, Belgium. The test on *D. magna* was performed according to the ISO (International Organization for Standardization) 6341, the test on *B. calyciflorus* following the ASTM (American Society for Testing and Materials, 1991) E1440-91, while the test on *T. platyurus* following the manufacturer procedure.

Prednisolone, dexamethasone and their photoderivatives were dissolved in dimethylsulphoxide (DMSO) and further diluted in double-deionized water to make the final stock solutions. The DMSO concentration in the exposure solutions, including controls, was 0.01% (v/v) that is a non-effect dose as estimated in preliminary tests. All bioassays were conducted under static conditions, with no renewal of the test solution, measuring dissolved oxygen and pH in each sample both at the start and at the end of testing. At the same time as toxicity testing, reference tests were performed with potassium dichromate (Aldrich) for all the organisms.

Juveniles (age, 0–2 h) of the rotifer *B. calyciflorus* were hatched from cysts after 16–18 h of incubation

under a light source of 3000–4000 lux at 25 °C in synthetic reconstituted medium (moderately hard medium EPA-600/4-85-013) and then exposed to the test sample. Hardness was 80–100 mg/l $CaCO_3$ and the dissolved oxygen content was at least 90% saturation at the beginning of the test. Tests were run in 36-well plates, five rotifers per well (0.3 ml of test solution, slightly different from the ASTM procedure), six replicates for each of the five concentrations. Test duration was 24 h, temperature 25 °C, in the dark. The test parameter considered was mortality and the concentration found to kill 50% rotifers in 24 h was indicated as LC50.

The bioassay on the anostracan crustacean *T. platyurus* was conducted using second- and third-instar fairy shrimp larvae hatched from cysts after 20–22 h of incubation at 25 °C in synthetic reconstituted freshwater (the same moderately hard EPA medium as rotifers) under continuous illumination (light source 3000–4000 lux). Tests were performed in 24-well plates, ten crustaceans per well (1.0 ml of test solution), three replicates for each of the five concentrations. Test duration was 24 h, temperature 25 °C, in the dark. The test parameter considered was mortality and the concentration found to kill 50% crustaceans in 24 h was indicated as LC50.

The test on *D. magna* Straus was performed using juveniles (age < 24 h), hatched from ephippia after 3–4 days of incubation at 20 °C under continuous illumination (light source 10 000 lux). The synthetic reconstituted freshwater, aerated before use, was the ISO hard medium (hardness 250 mg/l expressed as $CaCO_3$). Tests were performed with neonates < 24 h, five daphnids per vessel (10 ml of test solution), four replicates for each of the five concentrations. Daphnids were exposed to the samples at temperature of 20 °C in the dark. After 24 h the number of immobile daphnids was recorded to determine the sample concentration able to achieve 50% immobilization and it was indicated as EC50. *D. magna* was considered to be immobilized if it was not able to swim after gentle agitation of the liquid in 15 s of observation even if it can still move its antennae (ISO, 1996).

2.8. Chronic toxicity testing

The effects of the investigated drugs and their derivatives on the population growth inhibition were assessed using standard methods for chronic toxicity tests on the alga *P. subcapitata* (already known as *Selenastrum capricornutum*) and the crustacean *C. dubia*.

The algal growth inhibition test was run in 72 h according to the ISO procedure 8692 (ISO, 1987). The *P. subcapitata* inoculum (1×10^4 cells/ml) was taken from an exponentially growing pre-culture (strain CCAP 278/4) and poured in 25 ml of test solution in five concentrations and three replicates. Flasks were placed in a growth chamber at 25 °C under continuous illumination (8000 lux). The cell density was measured at 0 time and every 24 h for 3 days by an electronic particle dual threshold counter (Coulter Counter Z2, 100 μm capillary, Instrumentation Laboratory, Miami, FL, USA).

The test on *C. dubia* was run in 7 days and performed on young daphnids (<24 h old at the start of the exposure), obtained by acyclical parthenogenesis of individual adult females for at least three generations. The first females were born from the hatching of ephippia provided by MicroBioTests. Organisms were exposed individually to seven concentrations in beakers with 20 ml of synthetic reconstituted aerated hard ISO medium (total hardness 250 mg/l as $CaCO_3$) and the desired concentration of single compounds. Each treatment consisted of ten replicates per concentration incubated at 25 °C with a 16:8-h light: dark cycle (500 lux). Daphnids were fed daily with 100 μl of a suspension of the alga *P. subcapitata* (4×10^8 cells/ml), food fish (5 g/l) and yeast (5 g/l). Also test solutions were renewed daily as well as survival and offspring production assessed. From comparison between the number of offspring born from live or dead mothers at the end of the test in the sample batch and the control it was possible to calculate the concentration which gave rise to 50% population growth inhibition (ISO, 2001).

2.9. Data analysis

Raw data for all bioassays, except algal test, were analyzed using the Toxcalc™ (1996). For acute toxicity tests, the LC50s and EC50s were calculated by concentration/response regression using probit or trimmed Spearman–Karber method, as appropriate (Peltier and Weber, 1985). For the test with *C. dubia*, the value of the concentration that gave 50% population growth inhibition was calculated using Maximum Likelihood-Logit method. Raw test data from algae were analyzed by a Microsoft Excel 5.0 program (Phoenix, AZ, USA) tailored for this test. Algal growth inhibition (%) was calculated by integrating the mean values of cell density from t_0 to t_{72} h. Inhibition (%) values were tabulated against log-transformed data of concentrations to evaluate the test concentration corresponding to 50% algal growth inhibition.

3. Results and discussion

3.1. Phototransformation studies

Irradiation of an aqueous suspension of prednisolone (**2**) by a solar simulator for 4 h gave a complex mixture, which was resolved in its components by chromatographies. Along with unreacted prednisolone, the photoproducts **4**–**10**, identified by their spectroscopic features, were isolated.

The first compound was identified as the 5α-hydroxyderivative **4** by comparison of its spectral data (Appendix A) with those of the analogous photoproduct of prednisone (**1**) (DellaGreca et al., 2003). According to the structure the HRMS showed a molecular peak at m/z 378.2063 for the molecular formula $C_{21}H_{30}O_6$. Furthermore in the HMBC experiment the H-1 proton was correlated to the C-3, C-5 and C-10 carbons and the H-19 protons gave heterocorrelations with the C-5 and C-10 carbons.

Structure **5** was attributed to the second photoproduct. It had molecular formula $C_{21}H_{28}O_5$ according to the molecular peak at m/z 360.1915 in its HRMS spectrum. The ^1H and ^{13}C NMR resonance (Appendix A) were assigned by combination of COSY, TOCSY, DEPT, HMQC and HMBC experiments. The HMBC spectrum showed the correlations of the C-10 olefinic carbon with the H-2, H-4, H-6, H-7 and H-19 protons, as well as that of the C-3 carbonyl carbon with the H-22 and H-4 protons and that of the C-5 carbon with the H-4, H-6, H-7, H-9 and H-19 protons. The correlation in a TOCSY experiment between the H-4 and H-11 protons supported the presence of an ethereal bridge between the C-4 and C-11 carbons. The stereostructure of **5** derived from a ROESY experiment. The correlations with the H-18 methyl and the H-21 methylene protons allowed the assignment of the β-orientation to the H-12 protons at δ 2.20 and, consequently the α-one to the H-12 proton at δ 2.53. These nOe's pointed out a chair conformation of the C ring and the small couplings of the H-11 proton with the H-12 protons agreed with its α equatorial orientation. The correlation of the H-4 proton with the H-11 revealed its α-orientation and, consequently the β-one of the ethereal bridge. Finally the α axial orientation of H-19 methyl was supported by the nOe interaction of its protons with the H-4α proton.

The third compound was identified as **6**. It had molecular formula $C_{21}H_{30}O_6$ according to the molecular peak at m/z 360.1922 in the HR EIMS spectrum. The ^1H-NMR and ^{13}C-NMR data (Appendix A), compared with those of the corresponding photoderivative of prednisone, justified the structural assignment. As already verified on prednisone (**1**) (DellaGreca et al., 2003) the light caused also the degradation of the side chain at C-17. In fact the fourth photoproduct was identified as 11β-hydroxy-androsta-1,4-diene-3,17-dione (**7**) by comparison with an authentic sample obtained by MnO_2 oxidation of prednisolone. Structures **8**–**10** were attributed to the remaining compounds owing to the strong analogies of their physical features with those of **4**–**6**.

The photochemical behavior of dexamethasone (**3**) only partly matched that of prednisone (**1**) and prednisolone (**2**). In fact, the irradiation with the solar simulator for 8 h of its aqueous suspension converted dexamethasone (**3**) only in 15% amount and the photoderivatives **11** and **12** were isolated, without trace of the products obtained by degradation of the side chain at C-17. The data (Appendix A) of compounds **11** and **12** compared with those of **5** and **6**, justified the structure assignments.

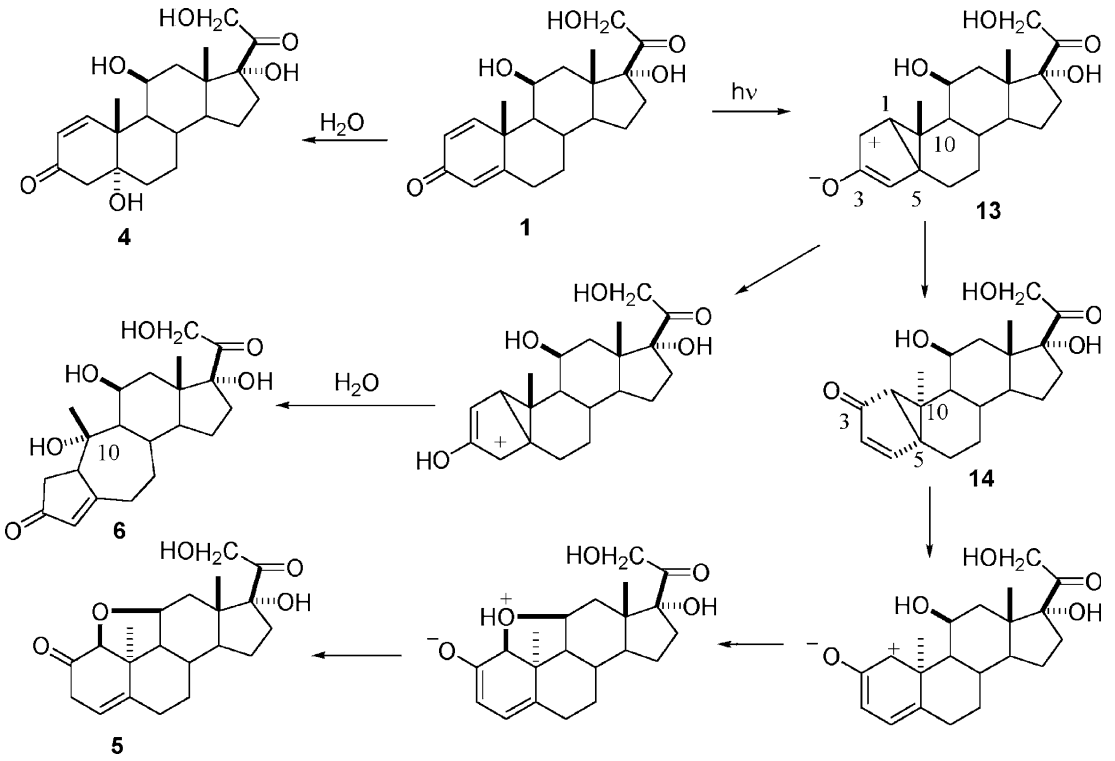
The formation of the 5α-hydroxyderivative **4** may be easily justified by a hydration reaction on the less hindered α-face of the Δ4 double bond (Scheme 1). The formation of the prednisolone photoderivatives **5** and **6** find explanation in the generally accepted mechanism for photoisomerization of cross-conjugated steroidal dienones (Williams et al., 1979). The light induced formation of cyclopropyl derivative **13**, protonation and subsequent attack of H_2O on the α-face generates **6**. Isomerization of **13** into the lumiprednisolone **14** followed by the attack of the hydroxyl group at C-11 on the C-4 position affords the ether **5**. The phototransformation of **1** into **2** by side chain degradation, and the same steps reported in Scheme 1 for prednisolone, justify the formation of **8**–**10**.

3.2. Toxicity studies

Acute toxicity data are reported in Table 1 for prednisolone and its photoproducts and in Table 2 for dexamethasone and the respective derivatives. Despite of the high concentrations tested, prednisolone **2** did not demonstrate a measurable value of LC50/EC50 except for rotifers. All the other compounds showed values by the order of units or dozen of mg/l except compound **9** slightly more toxic for the all organisms tested. Dexamethasone **3** and its derivatives **11** and **12** demonstrated such an activity as prednisolone photoproducts but also for these compounds the concentrations ranged from 10.88 to 60.11 mg/l. These order of concentrations should not be a problem because drug quantities found in the surface waters are usually below parts per billion (Aherne and Briggs, 1989; Raloff, 1998; Ternes and Wilken, 1999).

The long-term effects are shown in Tables 3 and 4. The detected drugs demonstrated a different toxic potential depending on the organism tested. Daphnids were found to be significantly more sensitive than algae. This result was particularly evident for the parental drugs where a median effective concentration of 0.23 mg/l was found for prednisolone on *C. dubia* against no effect at 160 mg/l for *P. subcapitata*. Also dexamethasone, while inhibited *C. dubia* 50% population growth at 0.05 mg/l, no effect showed at 100 mg/l on the algal growth. In this study algae showed median effective concentrations of the same order of magnitude than LC50 and EC50 found for acute toxicity tests. Compound **9** evidenced acute values for the all biota tested less than the chronic ones found for the algae.

Both the photoderivatives of prednisolone and dexamethasone showed effects on *C. dubia* that lead to long term action but, except compound **8** that was one



Scheme 1. Mechanism of phototransformation of prednisolone 1.

Table 1
Acute median effective concentrations in mg/l (95% confidence limits in brackets) of prednisolone and its phototransformation products

Compounds	*D. magna* (EC50)	*T. platyurus* (LC50)	*B. calyciflorus* (LC50)
2	NE[a] 85	23% mortal[b] 140	22.29 (20.82–24.56)
4	5.09 (3.98–6.54)	26.53 (18.44–38.13)	15.39 (12.58–18.84)
5	3.80 (2.70–5.33)	22.92 (17.16–30.61)	24.54 (20.82–28.92)
6	17.88 (14.06–22.74)	40.77 (25.18–66.01)	35.46 (30.46–41.29)
7	9.05 (7.20–11.37)	10.79 (8.52–13.67)	9.19 (5.52–15.23)
8	5.74 (4.81–6.85)	10.57 (8.21–13.59)	10.36 (7.47–14.37)
9	1.79 (1.38–2.32)	0.71 (0.5–1.0)	1.43 (1.14–1.81)
10	11.89 (9.78–14.46)	10.0 (7.58–13.18)	9.96 (8.46–11.74)

[a] NE = no effect at.
[b] Mortal = mortality at.

Table 2
Acute median effective concentrations in mg/l (95% confidence limits in brackets) of dexamethasone and its phototransformation products

Compounds	*D. magna* (EC50)	*T. platyurus* (LC50)	*B. calyciflorus* (LC50)
3	48.30 (39.91–58.45)	60.11 (44.21–81.73)	48.22 (41.37–56.20)
11	10.88 (7.28–16.26)	20.9 (16.49–26.50)	13.20 (11.43–15.23)
12	17.82 (13.84–22.94)	30.52 (25.54–46.66)	44.66 (38.91–51.25)

hundred times more active of prednisolone and compounds **9** and **10** ten times, other significant differences were not expressed. These chronic data differ from those of prednisone and its photoderivatives (DellaGreca

Table 3
Chronic median effective concentrations in ppm (95% confidence limits in brackets) of prednisolone and its phototransformation products

Compounds	*C. dubia* (7 days test)	*P. subcapitata* (3 days test)
2	0.23 (0.16–0.28)	NE[a] 160
4	0.22 (0.16–0.30)	27.46 (25.07–30.07)
5	0.12 (0.07–0.19)	30.42 (28.10–32.94)
6	0.22 (0.14–0.35)	24.65 (21.32–28.50)
7	0.51 (0.31–1.16)	14.14 (8.68–23.03)
8	0.007 (0.00026–0.026)	19.84 (17.98–21.88)
9	0.04 (0.018–0.06)	23.78 (11.75–48.13)
10	0.025 (0.014–0.038)	25.62 (20.32–28.90)

[a] NE = no effect at.

Table 4
Chronic median effective concentrations in ppm (95% confidence limits in brackets) of dexametasone and its phototransformation products

Compounds	*C. dubia* (7 days test)	*P. subcapitata* (3 days test)
3	0.05 (0.042–0.076)	NE[a] 100
11	0.13 (0.11–0.15)	12.15 (8.96–16.49)
12	0.06 (0.04–0.08)	40.75 (36.35–45.69)

[a] NE = no effect at.

et al., 2003) where no toxicity was found at concentrations harmful for the aquatic environment for prednisone compared with its photoproducts.

4. Conclusions

These laboratory studies evidence that prednisolone (**2**) and dexamethasone (**3**) are transformed by sunlight and enable to identify and to quantify the phototransformation products. Results from this study demonstrate that the chronic exposure to this class of pharmaceuticals causes inhibition of growth population on the freshwater crustacean *C. dubia* while the alga *P. subcapitata* seems to be less affected by the presence of these drugs. The low values of acute toxicity found for *B. calyciflorus*, *D. magna* and *T. platyurus* do not determine an acute environmental risk. Photoderivatives showed higher toxicity than parental compounds but the order of magnitude of effective concentrations was lower than drug quantities generally found in surface waters.

In conclusion the results give a strong indication on the importance of investigating the environmental fate of drugs and identifying their transformation products to assess their impact.

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Appendix A. Physical data of compounds *4–12*

Compound **4** had $[\alpha]_D$ +18.0° (c 0.5): EIMS m/z 378 $[M]^+$, 360 $[M\text{-}H_2O]^+$, 319 $[M\text{-}C_2H_3O_2]^+$; IR ($CHCl_3$): ν_{max} 3677, 3409, 1710 cm^{-1}; UV λ_{max} 231 nm; ^{1}H-NMR: δ (C_5D_5N) 7.78 (1H, d, $J = 5$, 6 Hz, H-1), 6.69 (1H, d, $J = 5$, 6 Hz, H-2), 5.26 (1H, d, $J = 19.2$ Hz, H-21), 4.82 (1H, d, $J = 19.2$ Hz, H-21), 4.72 (1H, brs, H-11), 3.20 (1H, d, $J = 19.2$ Hz, H-4), 3.12 (1H, m, H-16), 2.05 (1H, d, $J = 19.2$ Hz, H-4), 1.25 (3H, s, H-18), 1.20 (3H, s, H-19); ^{13}C-NMR: δ (C_5D_5N) 168.2 (C-1), 134.2 (C-2), 209.1 (C-3), 46.4 (C-4), 74.7 (C-5), 34.3 (C-6), 28.2 (C-7), 31.8 (C-8), 50.8 (C-9), 54.5 (C-10), 69.2 (C-11), 39.5 (C-12), 47.7 (C-13), 51.7 (C-14), 24.0 (C-15), 34.6 (C-16), 89.6 (C-17), 17.9 (C-18), 23.4 (C-19), 213.4 (C-20), 67.6 (C-21).

Compound **5** had $[\alpha]_D$ −159.0° (c 0.7): EIMS m/z 360 $[M]^+$, 342 $[M\text{-}H_2O]^+$, 301 $[M\text{-}C_2H_3O_2]^+$; IR ($CHCl_3$): ν_{max} 3685, 3505, 1727, 1710 cm^{-1}; UV λ_{max} 256 nm; ^{1}H-NMR: δ (C_5D_5N) 5.34 (1H, dt, $J = 2.1$ and 7.2 Hz, H-1), 5.27 (1H, d, $J = 19.2$ Hz, H-21), 4.82 (1H, d, $J = 19.2$ Hz, H-21), 3.11 (1H, ddd, $J = 2.1$, 4.1 and 15.2 Hz, H-2α), 3.02 (1H, ddd, $J = 3.1$, 11.6 and 14.7 Hz, H-16β), 2.62 (1H, dd, $J = 7.2$ and 15.2 Hz, H-2β), 2.53 (1H, dd, $J = 4.5$ and 14.5 Hz, H-12α), 2.20 (1H, dd, $J = 1.7$ and 14.5 Hz, H-12β). ^{13}C-NMR: δ (C_5D_5N) 116.6 (C-1), 38.4 (C-2), 204.3 (C-3), 86.6 (C-4), 54.0 (C-5), 26.2 (C-6), 26.9 (C-7), 31.2 (C-8), 54.3 (C-9), 145.6

(C-10), 77.9 (C-11), 32.8 (C-12), 48.2 (C-13), 49.1 (C-14), 22.9 (C-15), 34.2 (C-16), 89.3 (C-17), 17.5 (C-18), 25.0 (C-19), 213.0 (C-20), 67.5 (C-21).

Compound **6** had EIMS m/z 360 $[M]^+$, ^{1}H-NMR: δ ($CDCl_3$) 5.90 (1H, d, $J = 1.3$ Hz, H-4), 4.69 (1H, brs, H-11), 4.64 (1H, d, $J = 19.2$ Hz, H-21), 4.27 (1H, d, $J = 19.2$ Hz, H-21), 0.90 (3H, s, H-19), 1.15 (3H, s, H-18). ^{13}C-NMR: δ (CD_3OD) 58.3 (C-1), 37.3 (C-2), 212.8 (C-3), 131.5 (C-4), 188.3 (C-5), 32.1 (C-6), 30.9 (C-7), 40.4 (C-8), 62.7 (C-9), 76.8 (C-10), 70.3 (C-11), 40.3 (C-12), 40.3 (C-13), 53.7 (C-14), 26.0 (C-15), 34.7 (C-16), 90.8 (C-17), 18.7 (C-18), 20.4 (C-19), 213.8 (C-20), 68.1 (C-21).

Compound **7** had ^{1}H-NMR: δ (CD_3OD) 7.45 (1H, d, $J = 5.9$ Hz, H-1), 6.24 (1H, d, $J = 5.9$ Hz, H-2), 6.01 (1H, brs, H-4), 4.39 (1H, brs, H-11), 1.18 (3H, s, H-18), 1.52 (3H, s, H-19). ^{13}C-NMR: δ (CD_3OD) 161.0 (C-1), 128.0 (C-2), 189.5 (C-3), 123.0 (C-4), 174.9 (C-5), 35.0 (C-6), 33.1 (C-7), 32.5 (C-8), 57.5 (C-9), 46.8 (C-10), 71.1 (C-11), 42.0 (C-12), 46.8 (C-13), 53.2 (C-14), 23.7 (C-15), 37.0 (C-16), 214.4 (C-17), 17.5 (C-18), 22.5 (C-19).

Compound **8** had ^{1}H-NMR: δ ($CDCl_3$) 7.70 (1H, d, $J = 5.9$ Hz, H-1), 6.18 (1H, d, $J = 5.9$ Hz, H-2), 4.46 (1H, brs, H-11), 2.84 (1H, d, $J = 19.5$ Hz, H-4), 1.92 (1H, d, $J = 19.5$ Hz, H-4), 1.15 (3H, s, H-19), 1.09 (3H, s, H-18). ^{13}C-NMR: δ ($CDCl_3$) 167.1 (C-1), 134.8 (C-2), 209.9 (C-3), 45.9 (C-4), 74.8 (C-5), 33.9 (C-6), 26.8 (C-7), 31.0 (C-8), 51.8 (C-9), 54.3 (C-10), 69.6 (C-11), 40.2 (C-12), 47.0 (C-13), 51.5 (C-14), 23.2 (C-15), 35.5 (C-16), 219.3 (C-17), 16.1 (C-18), 21.7 (C-19).

Compound **9** had ^{1}H-NMR: δ ($CDCl_3$) 5.44 (1H, dt, $J = 2.1$ and 7.2 Hz, H-1), 4.45 (1H, brs, H-11), 4.18 (1H, s, H-4), 1.08 (3H, s, H-18), 1.40 (3H, s, H-19). ^{13}C-NMR: δ ($CDCl_3$) 116.4 (C-1), 37.9 (C-2), 204.1 (C-3), 86.4 (C-4), 53.9 (C-5), 25.7 (C-6), 25.2 (C-7), 30.5 (C-8), 54.7 (C-9), 144.8 (C-10), 76.7 (C-11), 33.0 (C-12), 47.4 (C-13), 48.9 (C-14), 25.2 (C-15), 35.3 (C-16), 219.7 (C-17), 15.6 (C-18), 21.0 (C-19).

Compound **10** had ^{1}H-NMR: δ (CD_3OD) 5.94 (1H, s, H-4), 4.68 (1H, m, H-11), 1.17 (6H, s, H-18, H-19). ^{13}C-NMR: δ (CD_3OD) 58.2 (C-1), 36.7 (C-2), 212.6 (C-3), 131.7 (C-4), 187.9 (C-5), 32.0 (C-6), 29.4 (C-7), 40.4 (C-8), 63.4 (C-9), 76.8 (C-10), 69.7 (C-11), 41.1 (C-12), 41.1 (C-13), 53.9 (C-14), 23.9 (C-15), 36.7 (C-16), 215.0 (C-17), 17.1 (C-18), 20.5 (C-19).

Compound **11** had ^{1}H-NMR: δ (CD_3OD) 5.45 (1H, dt, $J = 2.0$ and 7.0 Hz, H-1), 4.63 (1H, d, $J = 19.5$ Hz, H-21), 4.27 (1H, d, $J = 19.5$ Hz, H-21), 4.21 (1H, s, H-4), 4.19 (1H, m, H-11), 3.18 (1H, m, H-6), 3.02 (1H, m, H-16β), 3.00 (1H, m, H-2), 2.75 (1H, dd, $J = 7.0$ and 15.6 Hz, H-2), 2.38 (1H, brs, H-6), 2.25 (3H, m, H-7, H-12 and H-14), 1.80 (1H, m, H-12), 1.65 (1H, m, H-15), 1.53 (1H, m, H-7), 1.37 (3H, s, H-19), 1.17 (1H, m, H-15), 0.92 (4H; 1H, m, H-8; 3H, d, $J = 5.6$ Hz, H-22), 0.90 (3H, s, H-18). ^{13}C-NMR: δ (CD_3 OD) 116.8 (C-1), 38.0 (C-2), 203.5 (C-3), 85.7 (C-4), 53.0 (C-5), 25.1 (C-6), 30.0 (C-7), 36.2 (C-8), 99.7 (C-9), 143.8 (C-10), 98.1 (C-11), 31.2 (C-12), 49.6 (C-13), 42.5 (C-14), 21.2 (C-15), 34.3 (C-16), 90.1 (C-17), 17.0 (C-18), 20.5 (C-19), 212.1 (C-20), 67.8 (C-21), 14.9 (C-22).

Compound **12** had ^{1}H-NMR: δ (CD_3OD) 5.98 (1H, s, H-4), 4.62 (1H, d, $J = 19.1$ Hz, H-21), 4.52 (1H, m, H-11), 4.29 (1H, d, $J = 19.1$ Hz, H-21), 3.09 (1H, m, H-16β), 3.01 (1H, m, H-2), 2.65 (1H, m, H-2), 2.51 (2H, m, H-6 e H-6′), 2.51 (1H, m, H-7), 2.38 (1H, m, H-12), 2.21 (1H, m, H-14), 1.77 (2H, m, H-1, H-7), 1.68 (1H, m, H-15), 1.46 (1H, m, H-12), 1.3 (1H, m, H-15), 1.25 (1H, m, H-8), 1.22 (3H, s, H-19), 1.00 (3H, s, H-18), 0.89 (3H, d, $J = 17.2$ Hz, H-22). ^{13}C-NMR: δ (CD_3OD) 50.3 (C-1), 37.2 (C-2), 212.5 (C-3), 131.8 (C-4), 187.9 (C-5), 32.7 (C-6), 35.2 (C-7), 41.6 (C-8), 103.8 (C-9), 79.1 (C-10), 72.4 (C-11), 40.4 (C-12), 49.9 (C-13), 45.8 (C-14), 25.3 (C-15), 37.2 (C-16), 92.6 (C-17), 18.3 (C-18), 20.6 (C-19), 213.2 (C-20), 68.5 (C-21), 15.9 (C-22).

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