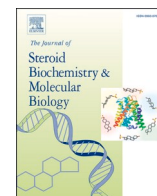




Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsmb

Ecdysteroid metabolism in mammals: The fate of ingested 20-hydroxyecdysone in mice and rats

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ARTICLE INFO

Keywords:

Anabolic steroid
Entero-hepatic cycle
Pharmacokinetics
Phytoecdysteroid
Rodent
Steroid metabolism

ABSTRACT

Phytoecdysteroids are molecules derived from sterol metabolism and found in many plants. They display a wide array of pharmacological effects on mammals (e.g. anabolic, anti-diabetic). Although these effects have been long established, the molecular targets involved remain to be identified. Like endogenous steroid hormones and bile acids, which are biochemically related, ingested or injected phytoecdysteroids undergo a set of reactions in mammals leading to the formation of numerous metabolites, only some of which have been so far identified, and it is presently unknown whether they represent active metabolites or inactivation products. In the large intestine, ecdysteroids undergo efficient 14-dehydroxylation. Other changes (reductions, epimerization, side-chain cleavage) are also observed, but whether these occur in the liver and/or large intestine is not known. The purpose of this study was to investigate the pharmacokinetics of 20-hydroxyecdysone (20E), the most common phytoecdysteroid, when administered to mice and rats, using, when required, tritium-labelled molecules to permit metabolic tracking. Bioavailability, the distribution of radioactivity and the kinetics of formation of metabolites were followed for 24–48 hours after ingestion and qualitative and quantitative analyses of circulating and excreted compounds were performed. In mice, the digestive tract always contains the majority of the ingested 20E. Within 30 min after ingestion, 20E reaches the large intestine, where microorganisms firstly remove the 14-hydroxyl group and reduce the 6-one. Then a very complex set of metabolites (not all of which have yet been identified) appears, which correspond to poststerone derivatives formed in the liver. We have observed that these compounds (like bile acids) undergo an entero-hepatic cycle, involving glucuronide conjugation in the liver and subsequent deconjugation in the intestine. Despite the very short half-life of ecdysteroids in mammals, this entero-hepatic cycle helps to maintain their plasma levels at values which, albeit low ($\leq 0.2 \mu\text{M}$), would be sufficient to evoke several pharmacological effects. Similar 20E metabolites were observed in mice and rats; they include in particular 14-deoxy-20E, poststerone and 14-deoxypoststerone and their diverse reduction products; the major products of this metabolism have been unambiguously identified. The major sites of metabolism of exogenous ecdysteroids in mammals are the large intestine and the liver. The entero-hepatic cycle contributes to the metabolism and to maintaining a low, but pharmacologically significant, concentration of ecdysteroids in the blood for ca. 24 h after ingestion. These data, together with parallel *in vitro* experiments provide a basis for the identification of 20E metabolite(s) possibly involved in the physiological effects associated with ecdysteroids in mammals.

Abbreviations: 20E, 20-hydroxyecdysone; 20,26E, 20,26-dihydroxyecdysone; 14d20E, 14-deoxy-20-hydroxyecdysone; 6 α OH20E, 6 α -hydroxy-20-hydroxyecdysone; 6 α OH14d20E, 6 α -hydroxy-14-deoxy-20-hydroxyecdysone; Post, poststerone; 14dPost, 14-deoxypoststerone; HPLC, high-performance liquid chromatography; HPLC-MS/MS, high-performance liquid chromatography–tandem mass-spectrometry; TIC, total ion current.

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<https://doi.org/10.1016/j.jsmb.2021.105896>

Received 29 January 2021; Received in revised form 31 March 2021; Accepted 31 March 2021

Available online 2 April 2021

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1. Introduction

Phytoecdysteroids evoke many pharmacological effects in mammals, including muscle anabolism [1], adipose tissue hypertrophy modulation [2,3] and osteoporosis prevention [4]. Studies in humans have shown that these molecules increase muscle mass and physical performance [5]. Ecdysteroids are also non-toxic to mammals [6] with an LD₅₀ for ingested 20-hydroxyecdysone (20E) in mice of >9 g/kg an LD₅₀ of 6.4 g/kg for i.p. injected 20E. The results of numerous animal studies conducted *in vivo* were obtained after ingestion or injection of various doses of phytoecdysteroids, resulting in circulating concentrations ranging between 10⁻⁸ to 10⁻⁶ M, but most often these concentrations have not been measured. As a consequence, some uncertainty remains concerning the active doses and how to translate the available *in vitro* data to *in vivo* situations. The molecular targets of ecdysteroids in mammals are still a matter of debate, and it has not yet been established whether their effects are only due to the originally administered molecules, or to some of their metabolites, which moreover have not been fully identified. For a better understanding of the mechanism of ecdysteroid action and evaluating a possible pharmacological use, it appears essential to study their bioavailability and metabolic fate in a range of mammalian species.

In non-human animals, it is possible to use radioactive molecules, which greatly facilitates their monitoring and metabolic fate. The metabolism of ecdysone, the only commercially available radioactive molecule when the study was made, was investigated in mice after intraperitoneal injections of the radioactive molecule [7]. Ecdysone levels increased transiently in the liver and then accumulated in the intestine, which, within one or two hours after injection, contained almost all the radioactivity. One day after injection, most of the radioactivity had been eliminated from the body. Moreover, the metabolism in mice of the most common phytoecdysteroid, 20-hydroxyecdysone (20E), was faster after intraperitoneal injection than after ingestion [8]. As with ecdysone, and whatever the mode of administration, radioactivity was rapidly taken up by the liver and then excreted into the intestine via the bile [8]. Excretion of phytoecdysteroids is primarily faecal in mice [8,7], while in humans and calves, urinary excretion seems somewhat more significant [40,9], even though the faecal content had not been quantified in these earlier studies and small amounts were recovered from urine as compared to the administered dose. The distribution of radioactivity from ³H-labelled 20E after injection into the caudal vein of mice has been studied, but no information was provided about the associated metabolism [10].

In humans, only four 20E pharmacokinetic studies have been performed. Simon and Koolman [11] showed that ingestion of ca. 15 mg 20E induced a urinary peak level of approximately 0.5 µM. Significant immunoreactivity was detected during the first 8 h following intake, but weak urinary levels were still observed until 24 h after ingestion [12]. After a single oral intake of 20 mg 20E [9], a more detailed study by RIA and LC-MS showed the existence in urine of a peak of detectable ecdysteroids after 3–4 hours and a total quantity of 1–2 mg, the exact nature of the compounds was not fully determined, although one could be tentatively identified as 14d20E. Finally, in another study [13], the ingestion of 434 mg 20E resulted in the urinary excretion of a total of 5 mg of 20E, corresponding to approximately 1% of the ingested amount.

In vertebrates, endogenous steroid hormones undergo oxidations, reductions and conjugations. Likewise, administered ecdysteroids are extensively metabolized. In mice, injected ecdysone undergoes dehydroxylation at position 14, probably by intestinal bacteria. Such a reaction, which is reminiscent of what happens to bile acids, is carried out by prokaryotes only [7]. When a larger amount of this molecule is injected, in addition to the production of 14-deoxyecdysone, a reduction in the B-ring of the 6-oxo group and epimerization of the hydroxyl at C-3 are also observed, which probably take place in the liver, according with what is known of the metabolism of sterols, steroids and bile acids, but this assumption still requires direct demonstration [14]. In mice, the metabolism of 20E is more complex because it can also involve cleavage

of the side-chain between C-20 and C-22, resulting in the formation of poststerone (Post) and its 14-deoxy [14α-H] derivative [15]. The demonstration of this reaction required the prior preparation of 20E tritiated on the nucleus (former experiments with 20E tritiated in positions 23 and 24 had revealed the formation of volatile radioactive compounds arising after cleavage of the side-chain). As well as in mice, the formation of 14-deoxy-20-hydroxyecdysone (14d20E) from ingested 20E was also observed in humans and calves [9,16].

Structure-activity relationship studies have shown that, when tested on the same target, the intensity of phytoecdysteroid effects varies quantitatively but not qualitatively, depending on their chemical structure [17]. Given the extensive metabolic conversions observed *in vivo*, this raises the question whether all the effects are due to the administered molecules or part, or all, of them to some of the metabolite (s). The direct activity of 20E appears likely, based on *in vitro* studies, although the absence of its conversion was not assessed by the authors [1,18], and the activities of its major *in vivo* metabolite 14d20E has not been tested *in vitro*, although it has recently been demonstrated that Post has anabolic activity in rats [19] and can activate protein kinase B (Akt) [20].

The objective of this study was to investigate the pharmacokinetics of orally administered 20E in mice and rats and to analyse over the following 24 h the kinetics of distribution and biotransformation in different organs. Metabolism taking place particularly in the liver and in the intestine has also been studied. A major aspect has been the analysis of the circulating levels of native molecules and their metabolites. The latter information is essential to define (1) a "physiological" range of concentrations to be used for *in vitro* tests and (2) which metabolites circulate and should therefore be tested *in vitro*. The anabolic activity of ecdysteroids is attracting medical attention for the treatment of conditions such as sarcopenia. It is already recognised that ecdysteroids possess several advantages over androgenic anabolic steroids for this condition, as the anabolic effects occur without the need for simultaneous exercise and without the associated negative physical and mental side-effects (reviewed in Dinan & Lafont [21]).

2. Materials & methods

2.1. Experimental animals

All experiments on animals were reviewed by the relevant institutional Ethical Committee and carried out in accordance with the European animal care guidelines (ETS #123; Directive 2010/63/EU) concerning the care and the ethical treatment of animals in research. C57BL/6J male mice (10 weeks old; Harlan Laboratories, Gannat, France) and Wistar or Sprague-Dawley rats (6–7 weeks old for ADME experiments and 10 weeks old for bile collection experiments; Charles River, l'Arbresle, France) were used for the different studies. Rats and mice were kept at 22 °C with a 12 h/12 h light/dark cycle.

2.2. Preparation of [³H]20-hydroxyecdysone and of non-radioactive reference ecdysteroids

20E (purity 99.7 %) was provided by Dr. Juraj Harmatha (Prague, Czech Republic) or purchased from NutraGreen (Shanghai, China) and purified to a final purity of 97.1 % by Patheon (Regensburg, FRG). The synthesis of the different reference compounds [14-deoxy-20-hydroxyecdysone (14d20E), poststerone (Post), 14-deoxy-poststerone (14dPost)] and 6αOH20E has been previously described [15].

The preparation of 20E tritiated in positions 1α,2α (specific activity = ca. 13.5 Ci/mmol; purity ≥90 %), has been previously described [15]. [1α,2α-³H₂]20E was diluted in a solution of non-radioactive 20E (see relevant method for details). In order to prepare a larger amount of radioactive 20E, a [5β,7,9α-³H₃]20E (2.4 Ci/mmol, 99.5 % radiochemical purity) was prepared from unlabelled 20E by exchange in ³H₂O under alkaline conditions (as initially described by Russell and Price

[22]) by Moravek Inc. (Brea, CA92821, USA) and stored in ethanol at -20°C . It contained tritium atoms in positions 5, 7 and 9, as shown by NMR analysis of a deuterated 20E prepared under the same conditions in D_2O (data not shown).

2.3. Radioactivity measurements

Radioactivity was measured by liquid scintillation counting (Beta-5, Kontron Instruments, Montigny-le Bretonneux, France) of 50 μL aliquots mixed with 2 mL scintillation cocktail (Ecoscint, National Diagnostics, Atlanta, GA). In the case of HPLC analyses, when entering the radioactivity monitor (LabLogic β -RAM), the effluent was mixed with scintillation cocktail (3 mL/min). The comparison with the UV traces allowed identification of the major metabolites.

2.4. Radio-HPLC analyses

To aliquots of each sample, ca. 5 μg of each of the key reference compounds (20E, 14d20E, Post and 14dPost) were added as markers. Samples were analysed by HPLC (Spectra Series P200, Thermo Separation Products, Brea, CA, USA) equipped with a radioactivity monitor (β -RAM, Lab Logic, Sheffield, UK). RP-HPLC used a C18 column (150 \times 4.6 mm) (ACE, Aberdeen, Scotland) eluted with a linear gradient of acetonitrile (10–40 % in 30 min, then 40–100 % in 3 min, 5 min 100 % and back to 10 % in 1 min) in water containing 0.1 % trifluoroacetic acid. The flow-rate used was 1 mL/min and photometric detection was set at 254 nm (Spectra Series UV100, Thermo Separation Products, Brea, CA, USA). To confirm the identity of some components, some samples were also analysed by normal-phase HPLC using an ACE 5 SIL column (150 \times 4.6 mm) eluted with a mixture of dichloromethane-isopropanol-water (DIW 125:25:2 v/v/v), or with a mixture of cyclohexane-isopropanol-water (CIW 100:23.5:1.2 v/v/v).

2.5. HPLC-MS/MS analyses

Ethanol extracts of stomach, small and large intestines, faeces and bile were dried and suspended in water before being purified on Sep-Pak® (C18 cartridges, Waters, Milford, MA), dried and redissolved in ethanol. Liquid chromatography-mass spectrometry (LC-MS/MS) was performed using an API 3200® turbo ion-spray (ABSciex, Framingham, MA) in negative-ion mode. Chromatographic separation was achieved on an XBridge® Shield RP18 column, (3.5 μm , 3.0 \times 100 mm; Waters, Milford, MA, USA) at 300 $\mu\text{L}/\text{min}$. The mobile phase consisted of mixtures of solvent A (0.1 % trifluoroacetic acid in Milli-Q water) and solvent B (acetonitrile). The gradient program for HPLC analyses started with 82 % solvent A and 18 % solvent B for 1 min, increasing linearly to 30 % solvent B at $t = 3$ min, held for 2 min, then increasing to 80 % solvent B at $t = 6$ min, held for 1 min, decreased to 18 % solvent B at $t = 7.5$ min and finally held for 6.5 min. Transition m/z , retention times and limits of quantification are displayed in Table S1A.

Analysis of faecal extracts from rats which received 1000 mg 20E/kg/d was performed on an Agilent HPLC/DAD/MS System with a Fortis C18 50 \times 2.1 mm (5 μm particle size) column eluted at 0.3 mL/min with a linear gradient of 10–35 % ACN in 0.1 % (v/v) in H_2O over 25 min. The MS operated in positive-ion mode. Elutions were monitored at 254 nm and by TIC. Retention times and mass spectra of eluted sample components were compared to those of reference ecdysteroids.

Plasma samples were analysed on an Acquity HPLC-MS/MS system with Xevo-TQS mass spectrometer (Waters, Milford, MA, USA) on a Acquity UPLC CSH fluoro-phenyl column (1.7 μm , 2.1 \times 150 mm) at 60 $^{\circ}\text{C}$ eluted at 0.4 mL/min with 92 % $\text{MeOH}/\text{H}_2\text{O}$ (1:9 v/v) [Solvent A]/8 % acetonitrile [Solvent B] for 4.5 min, followed by a linear gradient to 80 % A/20 % B at 7.5 min, at which it was maintained until 8.5 min, before being returned to starting conditions. The electro-spray MS operated in negative-ion mode. Retention times and mass transitions of eluted sample components were compared to those of reference ecdysteroids

(Table S1B).

2.6. Enzymatic hydrolyses

To test for the possible presence of ecdysteroid conjugates, hydrolyses of some radiolabelled samples of liver, small intestine, faeces and bile were performed by incubating them overnight with either 1 mg β -glucuronidase (*Helix pomatia*, Nr. G-0751 or *E. coli*, Nr. G-5897, Sigma-Aldrich, St. Louis, MO) in 500 μL sodium acetate buffer (50 mM, pH 5.3) or 1 mg esterase (from porcine liver, Sigma-Aldrich, St. Louis, MO) in 500 μL borate buffer (50 mM, pH 8). After hydrolysis, the solutions were dried and then taken up in 100 μL water. Aliquots of these solutions were analysed by HPLC in the presence of reference compounds, with simultaneous monitoring of radioactivity.

2.7. Metabolic fate of ingested 20E in mice

C57BL/6J male mice, aged 10 weeks and weighing approximately 20 g, had access *ad libitum* to water and a standard diet. The solution of tritiated 20E (200 μL of a solution containing 100 μg 20E and 6 μCi [$1\alpha,2\alpha$ - $^3\text{H}_2$]20E) was administered by gastric intubation. Mice were placed in individual cages to allow the collection of faeces and urine. After anaesthesia, they were sacrificed by intracardiac blood puncture and dissected at increasing time intervals (5, 15, 30 min, 1, 2, 4, 8, 12, 18 and 24 h: $n = 2$ for each time point). A second set of experiments was conducted using 1 mg 20E containing 5 μCi or 100 μg 20E containing 50 μCi and with samples being collected at 1 h, 2 h, 4 h, 8 h and 24 h after gastric intubation. For both experiments, after blood centrifugation (3500 rpm for 12 min at 4°C), plasma volume was measured. Plasma and red blood cells were extracted with 1 mL ethanol. Organs (liver, oesophagus + stomach, small intestine, large intestine, kidneys + bladder, epididymal adipose tissue, spleen) were collected into Corning tubes containing 7 mL ethanol (30 mL in the case of carcasses). Faeces and urine were extracted with 3 mL and 30 mL ethanol, respectively.

In parallel to the above experiment, stomach, small and large intestines were collected from mice 1 h and 2 h (and for faeces, after 24 h) after gastric intubation of 1 mg non-radiolabelled 20E, and further treated for HPLC-MS/MS analyses.

2.8. Pharmacokinetics of increasing amounts of 20E by oral gavage

Twenty-four Wistar rats were allocated to four groups of three males and three females each. They received the 20E (97.1 % purity) in 0.9 % NaCl at 50 mg/kg by intravenous (bolus) administration or in 0.5 % methylcellulose at 100, 300 or 1000 mg/kg by oral (gavage) administration. The dose formulations were administered on a single occasion under a constant dosage volume of 10 mL/kg or 5 mL/kg for intravenous and oral administrations, respectively. Blood samples were collected at 0.083 h, 0.25, 0.5, 1, 2, 4, 8 and 24 h after intravenous injection or 0.25, 0.5, 1, 2, 4, 8 and 24 h after oral administration. All samples were analysed by HPLC-MS/MS to determine plasma concentration levels of 20E, Post, 14dPost, 14d20E, 6OH14dPost, 20RSPost, 7,8dH20E, 6OH20E and 6OH14d20E. There was no mortality, morbidity or adverse clinical signs prior to, or during the treatments. 20E, even at the highest dose, was well tolerated by the rats. The body weight of each animal was recorded once before the treatment period and on the day of administration. After the last blood sampling, all animals were anaesthetized by an intraperitoneal injection of sodium pentobarbital and euthanized by cervical dislocation.

2.9. Treatment of rats with large amount of 20E for identification of faecal metabolites

Female rats (3) and male rats (3) were maintained in separate cages and fed 20E at 1000 mg/kg/day for 28 days. Faeces were collected corresponding to d2–5, d6–8, d9–12, d13–15, d16–18, d20–22 and

d23–27 (50–60 g/collection pot). A portion (5 g) of each faeces sample was homogenised (Ultraturrax®) in ethanol (25 mL) and then sonicated for 1 h and then centrifuged (5 min). The pellet was re-homogenised and sonicated with a further lot of ethanol, and the two extracts combined. A small aliquot was removed and diluted with the same volume of water and used for HPLC/MS analysis. The bulk of each extract was rotary evaporated to dryness, taken up in methanol (2 mL) and stored at -20°C for later analysis.

2.10. Bioavailability and excretion study in mice

In C57BL/6 mice, 20E was administered either *per os* in water at the dose of 50 mg/kg body weight (volume of administration = 10 mL/kg) or by perfusion in saline into the penis vein at the dose of 5 mg/kg body weight (volume of administration = 10 mL/kg). Blood was collected at $t = 0.25$ h, 0.5 h, 1 h, 3 h, 6 h, 8 h and 24 h after administration from the retro-orbital sinus with heparinised capillaries and placed in Eppendorf tubes. Urine and faeces were collected at 0–8 h, 8–24 h and 24–48 h after ingestion and stored at -20°C until further analysis. Blood samples were centrifuged at 10,600 g for 5 min at 10°C and plasma samples were collected, frozen and stored at -80°C . Urine and faecal samples were purified and then analysed by LC–MS/MS.

2.11. Bioavailability and excretion study in rats

In Wistar rats, 20E was either administered *per os* in water (50 or 100 mg/kg body weight) or intravenously (5 or 50 mg/kg body weight). In another experiment with rats, $[1\alpha,2\alpha\text{-}^3\text{H}_2]20\text{E}$ (20 μCi) was administered *per os*. Urine and faeces were separately collected at 0–8 h, 24–32 h and 32–48 h after ingestion for analysis by HPLC.

A more extensive experiment was performed after oral application of $[5,7,9\text{-}^3\text{H}]20\text{E}$ (150 $\mu\text{Ci}/\text{rat}$) and urine and faeces were separately collected at various time points over 7 days. It was found that the acid conditions in the stomach promoted partial exchange of tritium with water (ca. 7% of the administered radioactivity), resulting in the presence of tritiated water in the urine (mainly) and faeces (to a much lesser extent). To obviate the artefactual co-quantification of tritiated steroid and tritiated water, the samples were dried to evaporate off the tritiated water, allowing the tritiated non-volatile steroid to be accurately quantified. As a consequence, the use of $[5,7,9\text{-}^3\text{H}]20\text{E}$ was discontinued.

2.12. Extraction and purification of mouse and rat samples

In studies with mice, the ethanol extracts of plasma and red blood cells were centrifuged, evaporated to dryness and taken up in 1 mL ethanol. All organs, except the carcasses, were ground with an Ultraturrax®. The homogenates obtained were centrifuged (5000 rpm for 15 min) and supernatants were dried in a rotary evaporator. The residues were taken up in 2 mL methanol. Carcasses were finely cut and ground in 100 mL ethanol. The homogenates were centrifuged (5000 rpm for 15 min) and supernatants were collected and dried in a rotary evaporator. The solids obtained were taken up in 5 mL ethanol, sonicated and filtered through glass fibre filters (Whatman GFC®). The solutions obtained were dried and then taken up in 5 mL methanol. Faeces were ground, extracted in ethanol and centrifuged. The resulting supernatants, as well as urine samples, were dried, and then taken up in 2 mL methanol. Some lipid-rich faecal samples were purified by a chloroform-water (1:1 v/v) partition and the aqueous phase was analysed.

2.13. Bile secretion experiments

Bile secretion in male Wistar rats was studied either with unlabelled or tritiated compounds. For experiments using non-radiolabelled ecdysteroids, after anaesthesia a catheter was introduced into the bile duct. 20E was reconstituted in saline and administered intravenously at

a dose of 10 mg/kg. The bile was collected every 10 min and was thereafter analysed by HPLC-MS/MS. After application of $[1\alpha,2\alpha\text{-}^3\text{H}_2]20\text{E}$, ethanol (500 μL) was added to each bile sample and aliquots were radio-assayed. Aliquots (50 μL) of each of the 13 timed samples collected during the 2 h-kinetic were pooled for analysis by RP-HPLC and RP-HPLC-MS/MS. Some samples were hydrolysed (see above) prior to HPLC to test for the presence of steroid conjugates.

2.14. Incubation of rat and mouse intestinal content with ecdysteroids

A 14-week-old C57BL/6 mouse and a 5-week-old Wistar rat were used in a fed state. After sacrifice, intestinal contents (in large intestine in the proximal part after caecum) of the rat or mouse were collected. The intestinal content of the rat was suspended in 20 mL PBS, which was aliquoted in 10 smoked glass tubes with plugs (thus approximately 2 mL per tube). The intestinal content of the mouse was suspended in 2 mL PBS which was aliquoted in 10 smoked glass tubes with plugs (thus approximately 200 μL per tube). A volume of 50 μL ecdysteroid solution (20E, tritiated [$\text{ca. } 0.06\mu\text{Ci } [1\alpha,2\alpha\text{-}^3\text{H}_2]20\text{E}$] or not [125 μg], dissolved in PBS) was added to the intestinal contents and they were incubated under agitation for 1 h, 2 h or 3 h for cold products and for 1 h or 2 h for tritiated products. At the end of the incubation, absolute ethanol (400 μL) was added to mouse samples. Rat samples were transferred into 10 mL Corning tubes and 4 mL ethanol were added. Non-radioactive samples were analysed by HPLC-MS/MS, and radiolabelled samples by HPLC coupled to on-line radioactivity measurement.

3. Results & discussion

3.1. The significance of ecdysteroids

Ecdysteroids are the steroid hormones of invertebrates, where they regulate moulting, metamorphosis and reproduction [23]. These ecdysteroids are referred to as zooecdysteroids, in contrast to the phytoecdysteroids, which are found in a small, but significant, proportion of plant species, sometimes at very high concentrations (1–2% of d.w.) [24]. Thus, mammals can be exposed to ecdysteroids in their diets, with phytophagous mammals being potentially exposed to much higher levels than insectivorous ones. Evidence has been accumulating over several decades that ecdysteroids have a number of pharmacological effects on mammals, almost all of which can be viewed as beneficial. For example, ecdysteroids possess an anabolic effect, which is independent of the need for training and of the physical and mental problems associated with androgen-related anabolics. This has resulted in a wide range of ecdysteroid-containing preparations available for humans (sportsmen, bodybuilders etc.) and animals (e.g. race-horses, pets and farm animals). While such ‘doping’ may not currently be illegal, studies are underway to determine how best to monitor their use [25]. Additionally, there are prospects that ecdysteroids might be used as anabolic agents in medical conditions such as sarcopenia [26]. Also, ecdysteroids are being used as elicitors in gene-switch and gene therapy systems to control gene expression in medical situations [21], where success will depend on temporal and spatial control of adequate concentrations of the elicitor, and the absence of interfering metabolites. In certain cases, it has been found that the mammalian cells respond to added ecdysteroids even in the absence of the transfected ecdysteroid receptor [27, 28]. For all these reasons, it is necessary to have a good qualitative and quantitative understanding of the distribution, metabolism and excretion of ecdysteroids in mammals after ingestion or injection of these compounds.

3.2. The pharmacokinetics of 20E

3.2.1. Plasma levels in male rats after ingestion or injection

Intravenous 20E is cleared from the plasma of male rats within 4 h, while gastric 20E is cleared from the plasma by 24 h at most. T_{max} values

were 0.05 h and 0.5 h for *i.v.* and *p.o.* applications, respectively, C_{max} (ng/mL) and AUC (ng*h/mL) were 8550 and 5671 for intravenous application and 68 and 382 for *per os* application. The calculated bioavailability is thus 0.67 %.

3.2.2. Bioavailability of 20E

Table 1 below summarises the pharmacokinetic data for 20E in mice and rats according to sex of the animal, route of application and vehicle. Calculated bioavailabilities are low (*ca.* 1%), but also variable in both mice and rats.

3.2.3. Pharmacokinetics, bioavailability and analysis of ecdysteroid excretion in male and female rats after oral administration

The current data indicate that bioavailability after *per os* application is slightly higher in female rats, but remains low in both sexes (Figs. 1 and 2)

3.3. Plasma levels of 20E in relation to oral dose

Measurement of plasma levels after oral application of increasing amounts of 20E shows that relative bioavailability reduces at higher doses (Fig. 3), indicating that the uptake from the gut is saturable. This might represent saturation of the uptake mechanism or lower proportional solubility of 20E at higher amounts as water is absorbed during passage through the gut (the maximal solubility of 20E in water at 25 °C is *ca.* 20 mg/mL). Similar data are obtained with females with slightly higher bioavailability and interindividual variability (data not shown).

3.4. Kinetic studies of intestinal transit after a single oral bolus 20E application to mice

Fig. 4 shows the kinetics of radioactivity transit along the mouse digestive tract after oral application of [$1\alpha,2\alpha\text{-}^3\text{H}_2$]20E. The digestive tract always contains the bulk of the radioactivity (>85 %) until it is excreted. It shows that, over the 24 h study, the ingested radioactivity decreases regularly in the oesophagus and stomach during the first 30 min after ingestion, and first accumulates in the small intestine, where the maximum radioactivity is reached after 30 min, and then in the large intestine, where the maximum radioactivity is observed 60 min after ingestion. Its level remains relatively high in the large intestine from 1 to 4 h post-ingestion. The radioactivity is then progressively excreted in the faeces from 4 h after ingestion, and the faeces represent the major route for the elimination of 20E (and its metabolites); see below. Liver, kidney + bladder, adipose tissue, spleen, carcass and urine only ever contain low amounts of radioactivity (total <10 %) at any time.

3.5. Route and rate of elimination after oral application of [$5\beta,7,9\alpha\text{-}^3\text{H}$] 20E to rats

After oral application of [$5\beta,7,9\alpha\text{-}^3\text{H}$]20E to rats, elimination of radioactively-labelled 20E and its metabolites is completed within 48 h (Fig. 5). The labelled molecule prepared by exchange in tritiated water has shown a significant exchange with water in the animal (*ca.* up to 7% of the administered radioactivity). As a consequence, samples had to be dried before counting (see Supplementary Fig. 2), and this radio-labelled

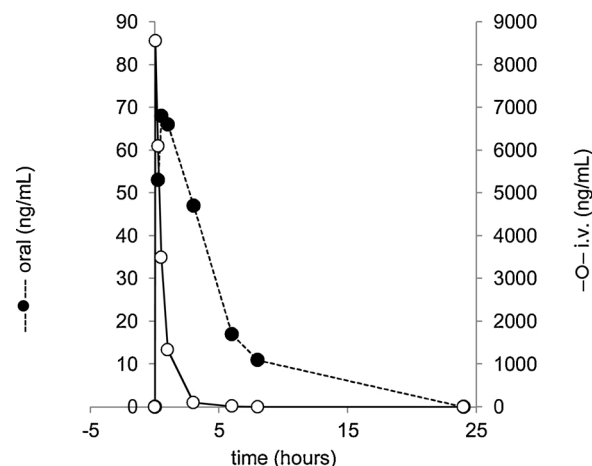


Fig. 1. Pharmacokinetics of 20E in plasma of male Wistar rats after intravenous (5 mg/kg in 0.9 % NaCl) or *per os* (50 mg/kg in water) application of the compound. 20E levels were quantified by HPLC/MS.

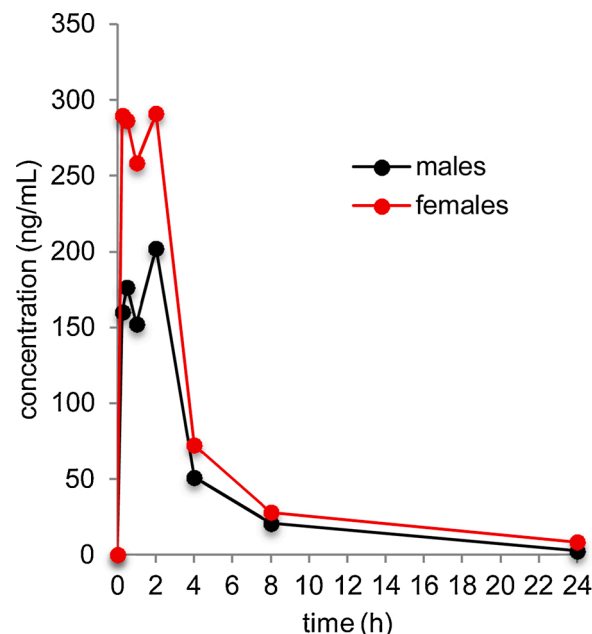


Fig. 2. Plasma 20E levels as measured by HPLC-MS/MS in male and female rats (Sprague-Dawley) treated *per os* with 100 mg 20E/kg in 0.5 % methylcellulose.

compound was not further used.

The amount of radioactivity recovered from the faeces is by far the major route of excretion, since the amount in the urine corresponds to only 1.40 % of the total radioactivity recovered.

Table 1

Pharmacokinetic parameters for plasma levels of 20E after application to mice and rats.

Species	Sex	Route	Dose (mg/kg)	Cmax (ng/mL)	AUC (ng*h/mL)	Route	Dose (mg/kg)	Cmax (ng/mL)	AUC (ng*h/mL)	Bioavailability (%)
Mouse	M	intravenous	5	10,095	1799	oral	50	19	77	0.43
	M	intravenous	5	1432	762	oral	50	171	106	1.39
	F	intravenous	5	1905	445	oral	50	57	91	2.04
	M	intravenous	5	1168	603	oral	50	114	98	1.62
	M	intravenous	5	8550	5671	oral	50	68	382	0.67
Rat	M	intravenous	50	173,000	40,500	oral	100	225	869	1.07
	F	intravenous	50	241,000	51,600	oral	100	316	1175	1.14

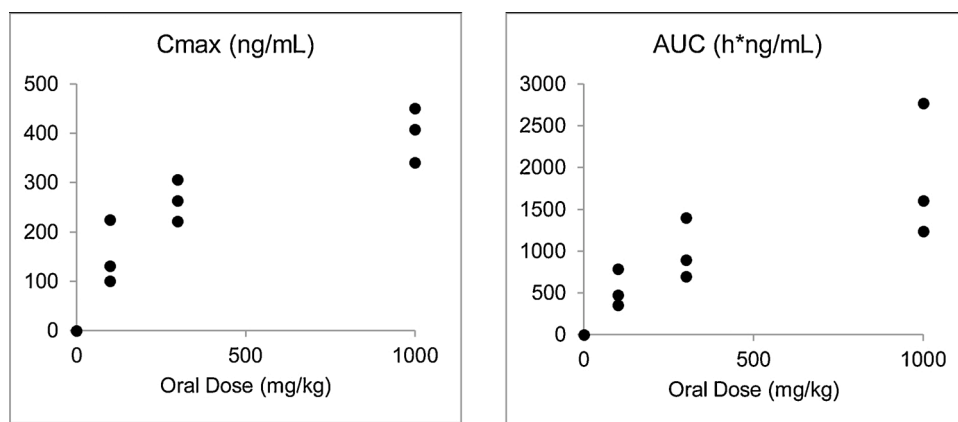


Fig. 3. Dose-response of C_{max} and AUC to oral 20E at 100, 300 and 1000 mg/kg in Sprague-Dawley male rats. Each data point corresponds to a mean of three animals.

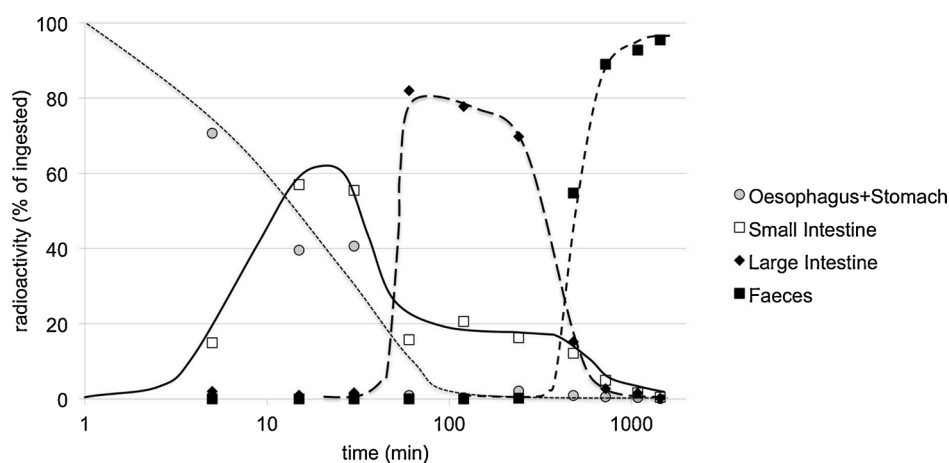


Fig. 4. Time-course of the distribution of radioactivity in the stomach/liver/intestine/faeces in mice after the oral application of $[1\alpha,2\alpha\text{-}^3\text{H}]20\text{E}$. Note the logarithmic scale for abscissa. Each value is a mean of 2 animals.

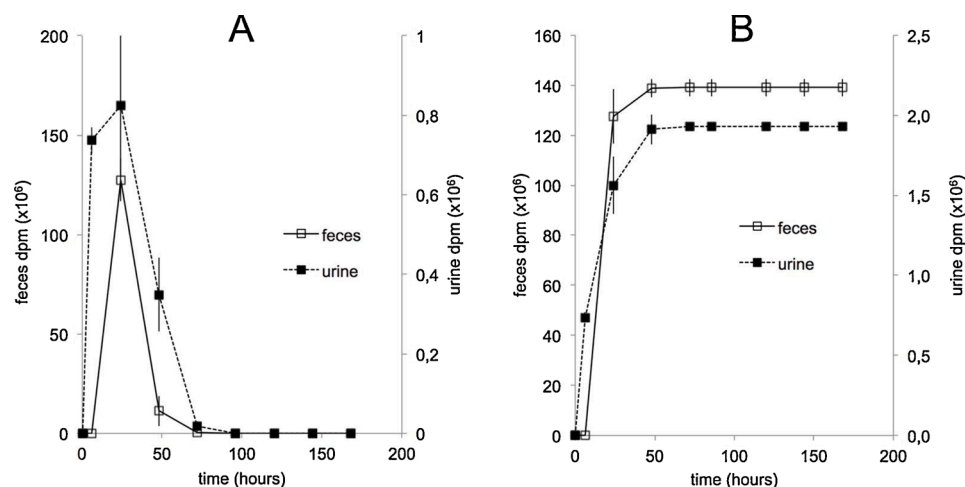


Fig. 5. The kinetics of $[5,7,9\text{-}^3\text{H}]20\text{E}$ elimination in faeces and urine after oral administration was measured over a 7-day period ($n = 6$). The graphs show the data after drying of the samples to remove tritiated water generated by isotopic exchange. A: elimination with time; B: cumulative elimination. See also Supplementary Figure S2.

3.6. Analyses of metabolites after $[1\alpha,2\alpha\text{-}^3\text{H}]20\text{E}$ ingestion in mice

No initial conversion of 20E occurs in the stomach/small intestine,

followed by early formation of 14d20E (observed at 30 min) in the large intestine. This clearly shows that 20E is not transformed by the very acidic conditions of the stomach (pH 2.98 in fed mice and 4.04 in fasted

mice, vs. 3.20 and 3.90 in rats, and 5.0 and 1.7 in humans, respectively [29]).

After >2 h, by contrast, complex metabolic profiles are observed in liver, small and large intestine extracts.

During the two hours following [$1\alpha,2\alpha\text{-}^3\text{H}_2$]20E ingestion in mice, RP-HPLC analyses showed that all tested organs, except the large intestine, contained mainly 20E (Fig. 6A and B). In the large intestine, HPLC revealed that 14d20E was already present 30 min after ingestion (Fig. 6D). It represented, respectively, 17 % and 50 % of the radioactivity in this organ 30 min and 1 h after ingestion (Figs. 6D and 6E).

Beyond two hours after ingestion, the ecdysteroid profiles of most organs became increasingly complex, and it was impossible to identify all the metabolites in each case (Figs. 6F-H). In liver, 20E was still detected 4 h after ingestion, accompanied by 14d20E and many other less polar compounds (Fig. 6B). In the large intestine, after 4 and 8 h, 14d20E was still detectable (Fig. 6H). From Fig. 6B and C, it appears that the small intestine and the liver do not metabolize 20E. As a consequence, the metabolites found in liver at 4 h (Fig. 6C) arise by reabsorption from the large intestine, and their simultaneous appearance in the small intestine results from their active secretion in the bile. This

provides evidence for an active enterohepatic cycle of 20E and its metabolites.

The HPLC profiles of liver and small intestine were more complex than those of the large intestine, and this was due to the additional presence of conjugates in the former that were hydrolysed in the large intestine. Similarly, faeces did not contain conjugates, and their HPLC profile remained unchanged after glucuronidase treatment (data not shown).

The identity of some of these metabolites was assessed by a combination of HPLC systems and comparison with synthetic reference compounds, and we found the same metabolites as in our previous studies using intraperitoneal injections [15]. The chemical formulae of the ecdysteroids discussed below are displayed in Supplementary Figure S1.

During the first two hours following ingestion of 20E, one sees essentially only 20E and 14d20E and it is only after this that the profile becomes complicated, without doubt from the point when Poststerone and/or 14d-Poststerone begin to be formed, presumably in a more distal part of the large intestine. Many compounds are detected by HPLC in the liver, intestines and faeces from 2 h after ingestion. In the liver, many unidentified compounds are found, which is consistent with the

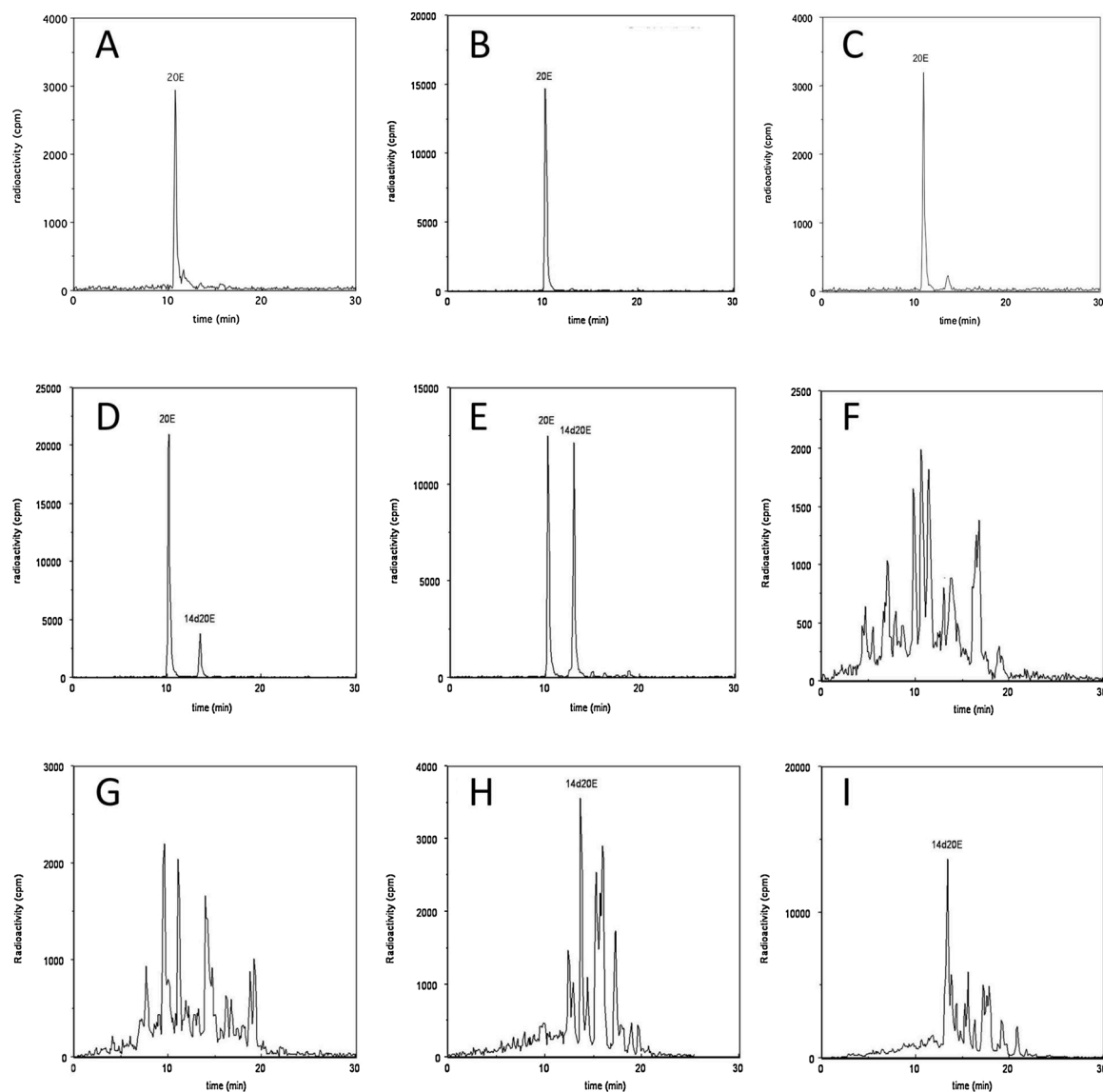


Fig. 6. Metabolism of [$1\alpha,2\alpha\text{-}^3\text{H}_2$]20E in mice monitored by RP-HPLC with radioactivity monitoring of extracts of organs at increasing time points after oral application. Give legends of A to I. A: plasma 1 h; B: small intestine 1 h; C: liver 1 h; D: large intestine 30 min; E: large intestine 1 h; F: liver 4 h; G: small intestine 4 h; H: large intestine 8 h; I: faeces 8 h-24 h.

reactions (reductions, epimerization at C3) previously observed for ecdysone [14,15].

3.7. Analyses of the bile secretion of ecdysteroids in rats

Previous metabolic studies of injected 20E [15] had shown that this molecule is rapidly transported to the intestine as a result of biliary secretion. Under these conditions, retention of significant plasma concentrations supposes the existence of an entero-hepatic circulation of these compounds, like that known for bile acids [30].

Experiments were performed to directly analyse ecdysteroid secretion in bile after intravenous administration of 20E (Fig. 7A). The biliary secretion of radioactivity after injection of [^{3}H]-20E strongly suggests the operation of a significant entero-hepatic cycle, as large amounts of radioactivity are found in the bile. Concentration peaks 20 min after administration, and secretion drops off sharply after 40 min. These observations provide a direct explanation for the high concentration of ecdysteroids in the intestine, whatever the mode of administration (*per os*, *i.p.* or *i.v.*).

An aliquot of pooled bile samples of [^{3}H]-20E showed the presence of a more polar metabolite on RP-HPLC ($R_t = 12$ min), which disappeared after glucuronidase treatment. In a parallel experiment using non radioactive 20E, HPLC-MS showed indeed the presence of a peak eluting just ahead of 20E with a MW consistent for a glucuronide of 20E (MW = 656; Fig. S3). It is not currently known which hydroxyl on the steroid is glycosylated.

The presence of hydrolysable conjugates of ecdysteroids in the liver and small intestine in mice suggests that conjugation takes place in the liver, the conjugates being thereafter secreted into the small intestine via the bile. The biliary secretion confirmed that conjugation occurred in liver. On the other hand, no conjugates were found in the faeces, a result

consistent with those of Kumpun et al. [15]. Such results are reminiscent of the phenomenon of hepatic conjugation of bile acids and steroids and their subsequent de-conjugation in the large intestine by the microbiome [31]. The ability of mouse liver microsomes (Sigma-Aldrich) to hydroxylate and/or glucuronidate [^{3}H]-20E was assessed *in vitro* in appropriate assays, but no conversion of either substrate in either assay could be detected (data not shown).

The absence of products of 14-dehydroxylation and side-chain cleavage in the bile indicate that these reactions do not occur in the liver.

3.8. The major circulating metabolites in rats following a single 20E oral administration (1000 mg/kg)

Fig. 8 summarises the levels of 20E and its metabolites (14d20E, Post, 14dPost) in plasma of male and female mice after gavage with 1000 mg 20E/kg. 6OH14dPost, 7,8dH20E, 6OH20E and 6OH14d20E were not detectable in any of the plasma samples over the 24 h following gavage with 20E. Low levels of 20,26E were observed that corresponded in fact to the small quantity already present in the administered 20E, and its amount did not increase with time.

Major sex differences in the plasma concentrations of Post, where higher levels are detected in males than in females (Fig. 8; Table 2). Thus side-chain cleavage occurs preferentially in males when they are first exposed to 20E. It is not currently clear if side-chain cleavage occurs in the animal's tissues or in the gut contents. Further, in animals which were daily fed 20E at 1000 mg/kg for 28 days and the metabolites quantified over the 24 h following the last gavage (Table 2; Fig. 8), the levels of Post, 14dPost, 14d20E are much higher, especially those of 14dPost in female plasma were 3- to 6-fold higher than in male plasma, which may reflect greater relative induction of side-chain cleavage activity in females, or better clearance of side-chain cleavage products

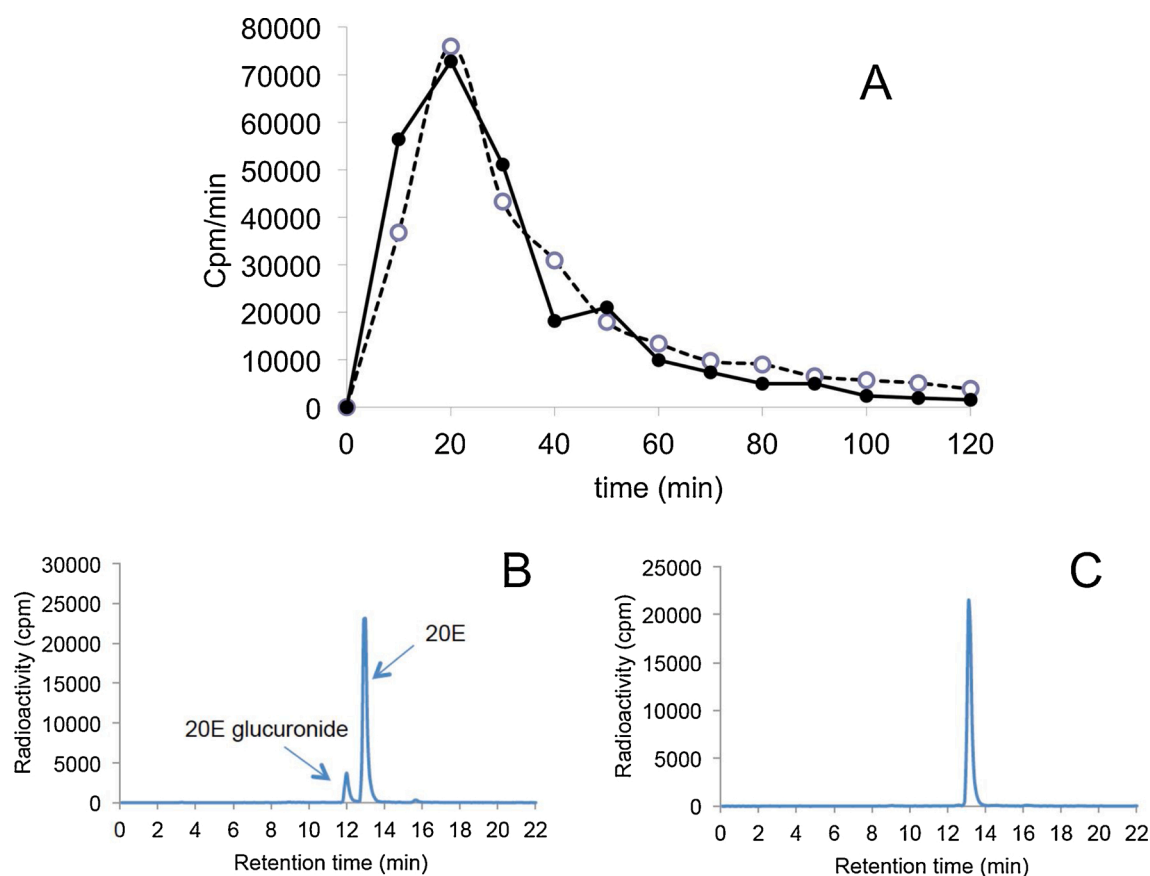


Fig. 7. Evidence for an efficient bile secretion (after intravenous injection). 7A represents the kinetics of bile secretion of two rats. The radioactive HPLC profile is shown before (7B) and after (7C) hydrolysis with glucuronidase (see also Supplementary Figure S3).

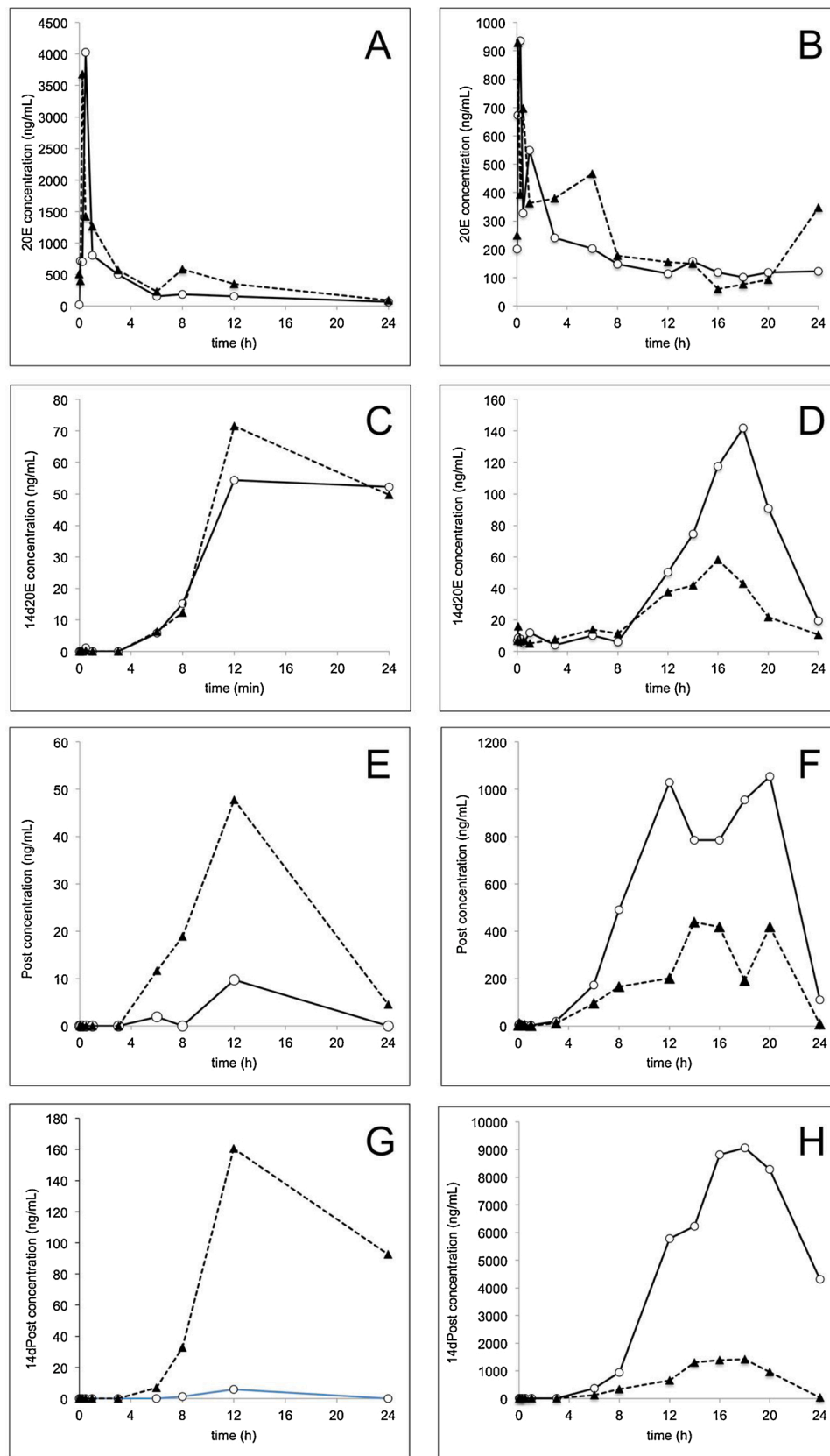


Fig. 8. Plasma 20E and its major metabolites over 24 h as measured by HPLC-MS/MS in male (dotted line) and female (full line) Wistar rats after oral feeding of 1000 mg 20E/kg at $t = 0$ h d1 (ACEG) and then at d28 after daily administration for 4 weeks (BDFH). A, B: 20E; C, D: 14d20E; E, F: Poststerone; G, H: 14-deoxypoststerone.

Table 2

AUC values for 20E and its metabolites in plasma of male and female Wistar rats after oral gavage of 1000 mg 20E/kg for 1 day or 28 days.

Ecdysteroid	Plasma AUC _{24h} (ng*h/mL)			
	Female Rats		Male Rats	
	D1	D28	D1	D28
20E	6568	4343	10,033	5583
Post	40	11,639	277	3930
14dPost	37	102,845	1583	14,508
14d20E	806	1204	925	587
20RSPost	Not detected	650	50	262

from the plasma in males. Induction of metabolism of 20E over the 28 days is supported by 2-fold lower levels of 20E in the plasma of both males and females on d28 relative to d1, while much higher levels of Post, 14dPost and 20RSPost are found in the plasma of both males and females at d28. Whether this reflects a modification/adaptation of the microbiome will deserve additional experiments.

In mice having ingested [$1\alpha,2\alpha\text{-}^3\text{H}_2$]20E, plasma contained only low amounts of radioactivity throughout the 24 h following ingestion. However, given the administered amounts, circulating levels were not negligible. During the first two hours after ingestion, only 20E is detected in plasma, and its concentration is 0.67 μM after an hour and 0.42 μM after two hours. Radioactivity measurements show that circulating levels were maintained at values ranging between 0.1–0.2 μM from 4 to 8 h after ingestion.

3.9. Rat faeces contain a complex array of metabolites after feeding with 1000 mg 20E/kg

Elution profiles for the early faecal extracts (d2–5 & d6–9) from

male and female samples monitored at 254 nm showed 4 major UV-absorbing peaks (e.g. Fig. 9, upper panel), while later samples showed additional (3–5) minor UV-absorbing peaks. The 4 initial major peaks were identified as 20E, Post, 14d20E and 14dPost on the basis of their retention times, UV-absorbance and mass spectra. The proportions of these peaks (in relation to the total UV absorbance profile at 254 nm) changed and reduced with time in the later faecal extracts as the later minor peaks appeared, but 20E and 14d20E remain the major components. In view of how clean the extracts are and their very high content of ecdysteroids, it is expected that each of the minor peaks is an ecdysteroid metabolite, but this needs to be confirmed by isolation and physico-chemical identification. TIC-monitoring of the same extracts reveals extra non-UV-absorbing components (12–15), all of which show the sequential loss of 18 amu (H_2O) units in their mass spectra, which is characteristic of the polyhydroxy ecdysteroids. The lack of significant UV-absorbance in these peaks indicates that the 6-oxo-7-ene-14-hydroxy chromophore has been modified. Comparison of the MWs of these peaks (Fig. 9, lower panel), which shows gains of 2 or 4 amu in relation to the UV-absorbing peaks, indicates that the most probable explanation are permutations of metabolites based on reduction of the 6-oxo group (to 6 α -OH and/or 6 β -OH) and/or reduction of the Δ^7 -double-bond (resulting in 8Ha and/or 8Hb), together with or without 14-dehydroxylation and/or side-chain cleavage. Moreover, the 20-oxo group of Post and 145dPost can also be reduced, leading to two 20R/20S metabolites.

Metabolism increases continuously during chronic administration. When rats are fed 20E on a daily basis and the metabolite profiles in faeces are assessed (Fig. 10), the proportions of 20E and the 3 UV-absorbing metabolites decreases with time (Fig. 10, panel A). Side-chain cleavage is hardly observable in day 1 in rats, but becomes more and more important on later days (Fig. 10, panel B).

The complete identification of all the faecal metabolites is ongoing and will be presented in a separate paper.

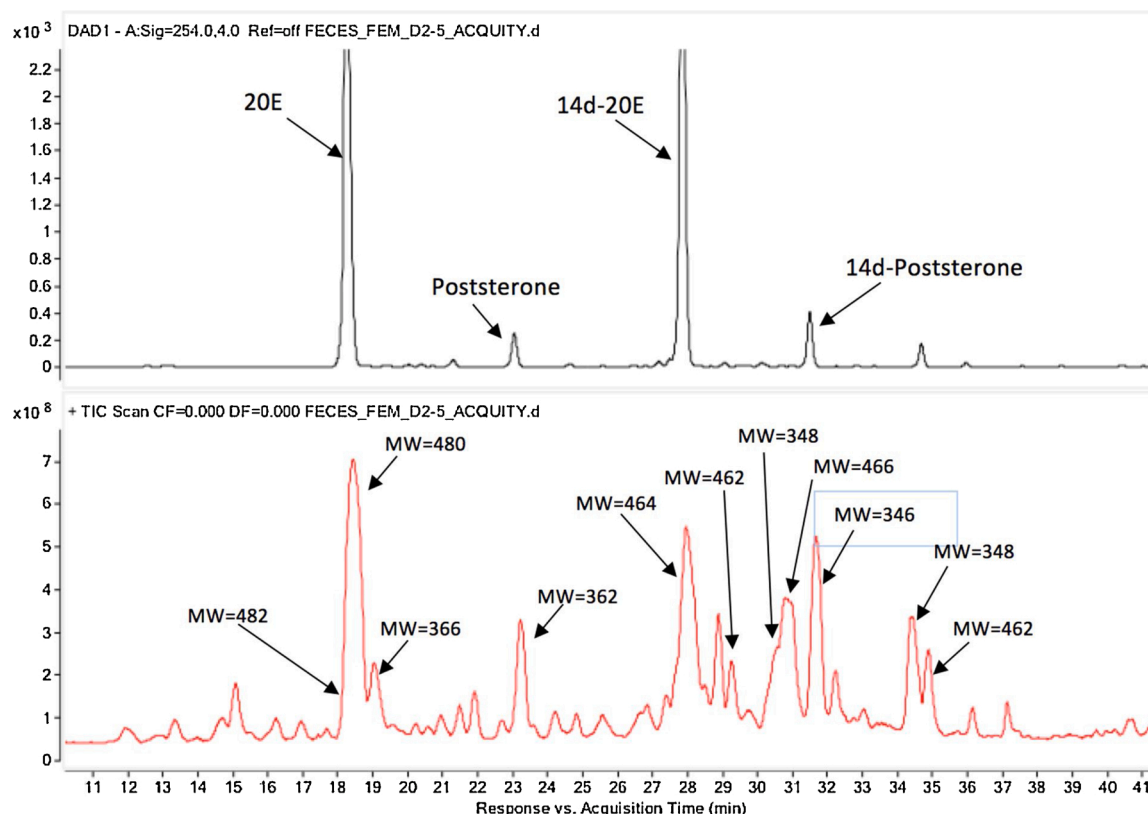


Fig. 9. Analysis by RP-HPLC/DAD/MS of faeces (from the same experiment as that of Fig. 8), showing 4 major metabolites which absorb UV (upper panel) and those plus a lot of additional ones that no longer absorb UV (reduced on B-ring; lower panel) detected in the TIC chromatogram. The data shown correspond to female rat faeces (d6–9 sample).

3.10. Evidence that 14-dehydroxylation is performed by the intestinal microbiote

Our results (Fig. 11) confirm that the conversion of 20E into 14d20E (and the conversion of Post into 14dPost) takes place in the large intestine, and is performed by intestinal microbiome. Such a result is consistent with what is known of the metabolism of bile acids [30] and, in particular, the de-hydroxylation in position-7 of cholic acid to form deoxycholic acid. Moreover, previous experiments showed the absence of 14d20E formation when 20E is fed to axenic rats [32].

No side-chain cleavage was observed, but we used naive rats and maybe using the microbiote of animals fed for 1–2 weeks would allow observation of this reaction. This point deserves additional studies.

3.11. Consideration of plasma concentrations of 20E and its metabolites and their implications

3.11.1. Plasma concentrations in relation to the administration route

The metabolic picture obtained in this study (Fig. 12) indicates a slower metabolism of ingested ecdysteroids than when they are injected, in line with data previously reported [7]. Our results with injected molecules show a rapid clearance from the blood. In the literature, it was reported that 20E, following injection, is rapidly eliminated from the blood (initial half-life of 8.15 min [33]). When using this value and considering an initial concentration of ca. 200 μM following injection, this would result in plasma concentrations below 0.01 μM after two hours, to be compared with the 0.42 μM found in the present experiments. This discrepancy is, however, not due to the mode of administration. Injections result in immediately very high concentrations, which lead one to consider the low levels detected after 1 or 2 h as "negligible", although, in fact, they remain physiologically relevant. By contrast, oral administration results in low plasma levels ($\leq 1 \mu\text{M}$), but those are sustained owing to a steady state between the continuous absorption from the intestine and the active secretion by the liver into the bile. Such sustained low levels were already observed in early experiments by Hikino et al. [8].

3.11.2. Plasma concentrations after oral application

Baseline plasma levels of phytoecdysteroids are usually very low, as they result from a low dietary intake [11]. In our study in mice using $[1\alpha,2\alpha\text{-}^3\text{H}_2]20\text{E}$, an intake of 100 micrograms of 20E resulted in a plasma concentration between 0.1 and 0.7 μM of 20E and metabolites for at least 8 h following ingestion. Hikino et al. [8] orally administered 700 μg of tritiated 20E, which resulted in circulating levels of the range of 1–2 μM up to 4 h after ingestion, which is quite consistent with our results. However, these same authors observed a late increase in plasma radioactivity 8 h after ingestion, which remains unexplained (but we should note that these concentrations are calculated from the radioactivity found in plasma, and the nature of circulating compounds was not determined in that study).

Concerning the rat ecdysteroid bioavailability data, in the literature it has been shown that a daily intake of 200 mg and 380 mg of 20E/kg body weight over 4 and 12 weeks, respectively, resulted in serum concentrations of 1.2 μM and 0.4 μM [26,34]. However, in these studies, we do not know at what moment the plasma was sampled, so it is difficult to compare these findings with our results.

In another study, also with rats, gavage feeding with up to 200 mg 20E/kg and measurement of plasma levels 30–240 min later confirms the low bioavailability of 20E, since levels were only 0.3–0.5 $\mu\text{g}/\text{mL}$ (i.e. ca. 0.6–1 μM) if the 20E was supplied in 3% DMSO in saline or 1.52 $\mu\text{g}/\text{mL}$ (3 μM) if supplied in Labrasol [35].

3.11.3. Metabolites in the plasma after oral application

In mice fed $[1\alpha,2\alpha\text{-}^3\text{H}_2]20\text{E}$, plasma contains mainly 20E for the first two hours after ingestion. After two hours, we were not able to identify clearly plasma metabolites by HPLC because of the small amount of radioactivity in this compartment. However, the situation in rats is clearer because of the larger size of the animals coupled with feeding much larger amounts of unlabelled 20E and sensitive and specific detection of metabolites by HPLC-MS/MS. It was not only possible to identify 14d20E, Post, 14dPost and 20RSPost as metabolites in the plasma, but also to follow their titres over the 24 h following oral application and to identify differences in their levels between males and female rats, apparently arising from differential side-chain cleavage activities between the sexes. Metabolites arising from 14-dehydroxylation and side-chain cleavage only start to arise after 8–10 h, when biliary secretion of 20E plays a significant role and passage through the gut brings the 20E to the large intestine.

3.11.4. Bioavailability in relation to the structure of the ecdysteroid

The low levels found in plasma indicate that the bioavailability of ingested 20E is low, probably because of its relatively high polarity (owing to the presence of six hydroxyl functions). The literature data show that, by comparison, ingested ecdysone (which has 5 hydroxyl groups) is found at markedly higher plasma concentrations as compared with 20E taken at the same dose [12]. It can therefore be expected that esterification (e.g. acetylation) of one (or more) hydroxyl functions could be used to improve the bioavailability of 20E. Such esterifications are currently used to prepare slow-release forms of steroids [36].

3.11.5. Which are the active metabolites?

Oral bioavailability of 20E is low in mice and rats. These low concentrations are nevertheless compatible with those ($\geq 0.1 \mu\text{M}$) of 20E sufficient to stimulate protein synthesis in myocytes [1]. In that case, we presume that 20E is the active factor, since our experiments using myocytes or hepatocytes in culture showed that it was not metabolized (unpublished data). On the other hand, the concentrations of 20E required to induce hypoglycaemic effects on hepatocytes or to stimulate the differentiation of keratinocytes were much higher (1–100 μM [18] or 200 μM [37], which raises doubts that 20E itself could produce the same

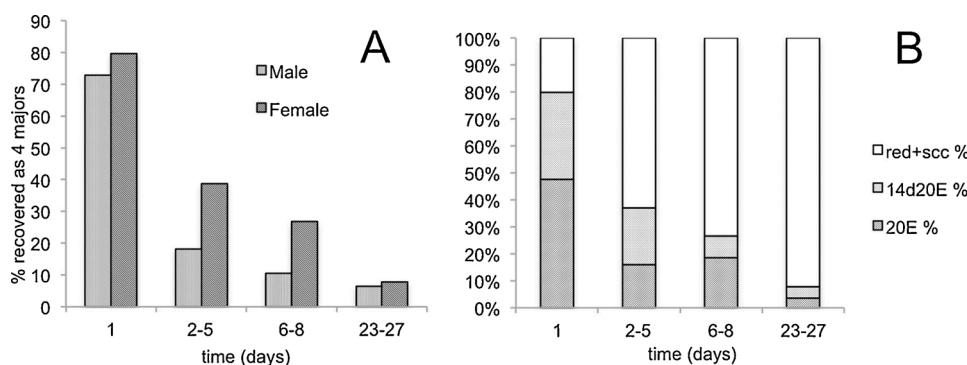


Fig. 10. The extent of 20E degradation increases during chronic administration. Rats were fed daily over 28 days with 20E (1000 mg/kg/d) and faeces collected over the denoted time periods, before extraction and analysis and quantification of metabolites by HPLC-MS/MS. Panel A shows the proportion of the recovered ecdysteroids in the form of the 4 initially major compounds (20E, 14d20E, Post and 14dPost) in male and female rats. Panel B shows the proportions recovered as 20E, 14d20E and reduced + side-chain cleavage products in the faeces of the female mice.

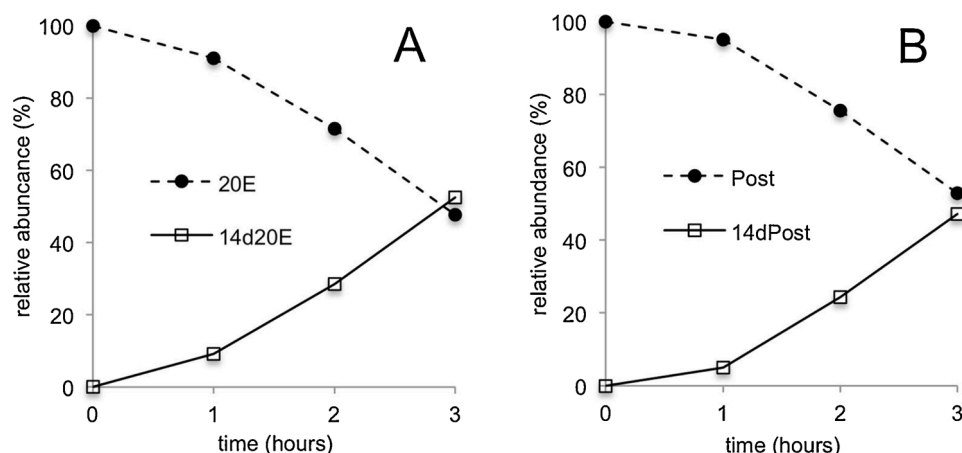


Fig. 11. Anaerobic metabolism (14-dehydroxylation) of 20-hydroxyecdysone (panel A) and poststerone (panel B) by the intestinal contents from rats.

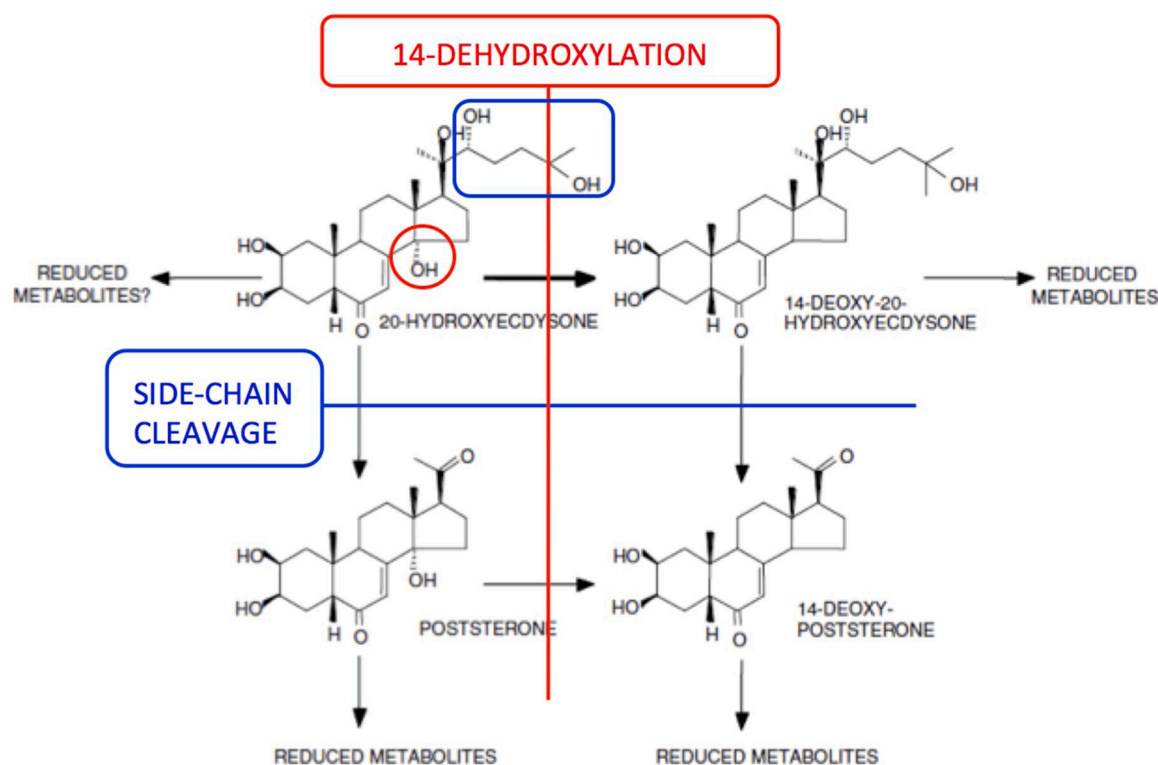


Fig. 12. Major metabolites of 20E in mice.

effects after *in vivo* administration. However, we do not know the possible effects of a chronic exposure to low doses of 20E over several weeks.

4. Future prospects

In the future, it will be necessary to study the impact of 20E formulation on its bioavailability which is already low. For example, what is the bioavailability of 20E when incorporated into a solid food, for example when administered within a quinoa extract, as recently tested in mice [2]? Despite a probably low bioavailability of 20E, low dosages as 5–10 mg/kg body weight are active in mice [2,38]. Similarly, despite a short half-life, the molecule provided once a day by gavage is able to evoke physiological modifications (but we still do not know whether its metabolites are active). Moreover, possible changes of biotransformations during chronic ingestion of 20E will be interesting to

study, in order to detect the possible induction of detoxification mechanisms (hepatic or other, due to possible modifications of the gut flora).

Rodents are useful mammalian models because of their experimental amenability and the greater range of acceptable investigative methods which can be used on them, but the findings cannot necessarily be fully extrapolated to humans, so it will be important to use the findings from these rodent experiments to structure experimental hypotheses to determine whether the findings are applicable to humans.

5. Conclusions

- 1 Low oral bioavailability is a limitation to the pharmaceutical application of 20E by this route.
- 2 High levels of orally supplied 20E are very well tolerated by rodents
- 3 There is no accumulation/storage of 20E in the organism

- 4 Oral 20E and its metabolites are totally eliminated from rats within 48 h, predominantly by the faecal route.
- 5 Significant levels of metabolic conversion begin only in the large intestine
- 6 Metabolism involves the gut microbiote (at least 14-dehydroxylation, but probably also side-chain cleavage).
- 7 There is very efficient bile secretion of 20E with no metabolism except for a low glucuronide formation.
- 8 The enterohepatic cycle contributes to maintaining and prolonging ecdysteroid levels in the plasma and promoting a complex series of metabolites deriving from Post, which can be recovered from the faeces.
- 9 Faecal analyses after feeding rats with large amounts of 20E provides and amenable and promising system to investigate the components of the complex metabolism.
- 10 A subsequent paper ([39] accepted for publication) will deal with poststerone and its complex liver metabolism in rodents.
- 11 These data provide a precise background for further experiments concerning 20E metabolism in humans.

Author contributions

L. Dinan: investigation, writing – original draft, review and editing
 C. Balducci: investigation
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 A.-S. Foucault: investigation
 A. Bakrim: investigation
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 J.-P. Girault: NMR analyses
 C. Tourette: investigation, writing – review
 W. Dioh: writing – review and editing
 P.J. Dilda: writing – review and editing
 S. Veillet: funding acquisition, writing – review and editing
 R. Lafont: conceptualisation, writing – original draft, review and editing

Funding

This study was funded by Biophytis SA, Paris, France, and the Fonds unique interministériel (SARCOB), France. LD, CB, A-SF, LG, CT, WD, PD, SV and RL are past or present employees of Biophytis. The Company did not have any influence on the study design, data interpretation or manuscript preparation.

Declaration of Competing Interest

None.

Acknowledgements

We thank Prof. Jean Mariani for his helpful comments on the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jsbmb.2021.105896>.

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