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The metabolism of 20-hydroxyecdysone in mice: Relevance to pharmacological effects and gene switch applications of ecdysteroids

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ABSTRACT

Ecdysteroids exert many pharmacological effects in mammals (including humans), most of which appear beneficial, but their mechanism of action is far from understood. Whether they act directly and/or after the formation of metabolites is still an open question. The need to investigate this question has gained extra impetus because of the recent development of ecdysteroid-based gene-therapy systems for mammals.

In order to investigate the metabolic fate of ecdysteroids in mice, [1α,2α-^{3}H]20-hydroxyecdysone was prepared and injected intraperitoneally to mice. Their excretory products (urine + faeces) were collected and the different tritiated metabolites were isolated and identified. The pattern of ecdysteroid metabolites is very complex, but no conjugates were found, in contrast to the classical fate of the (less polar) endogenous vertebrate steroid hormones. Primary reactions involve dehydroxylation at C-14 and side-chain cleavage between C-20 and C-22, thereby yielding 14-deoxy-20-hydroxyecdysone, poststerone and 14-deoxypoststerone. These metabolites then undergo several reactions of reduction involving, in particular, the 6-keto-group.

A novel major metabolite has been identified as 2β,3β,6α,22*R*,25-pentahydroxy-5β-cholest-8(14)-ene. The formation of this and the other major metabolites is discussed in relation to the various effects of ecdysteroids already demonstrated on vertebrates.

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1. Introduction

Ecdysteroids (insect moulting hormones) display many pharmacological effects in mammals (reviewed in [1–7]). For instance, 20-hydroxyecdysone (20E) has anabolic effects in mice and humans. Ecdysteroids (phytoecdysteroids) are available in large amounts (>1% of the dry weight) from certain plants [8], some of which (e.g. spinach, quinoa) are used for human food. As a consequence, ecdysteroid use has been developed by high-level sportsmen as a non-prohibited supplement ostensibly to improve performance. Over recent years their use has also become popular among bodybuilders, who ingest them as part of a more or less complex cocktail of anabolic substances to increase their lean muscle mass [5,7]; similar anabolic effects have also been reported for domesticated animals (e.g. sheep [9], quails [10] and pigs [11]).

The mechanism of this anabolic effect is not fully understood; ecdysteroids seem to both decrease protein breakdown and enhance protein synthesis at the translational level [12]. 20E stimulates in a dose-dependent manner [^{3}H]leucine incorporation into muscle proteins *in vitro* (e.g. [13]), but 20E is rapidly metabolized *in vivo*, and the possible effects of its metabolites are still unknown. In this respect, it is of considerable interest to determine the metabolic fate of 20E in mammals/humans. In addition, such a knowledge would facilitate a rational approach to demonstrate ecdysteroid intake by sportsmen or farm animals in the event that the use of ecdysteroids would become legally controlled [14–17].

There is considerable interest in the development and application of gene-switch systems, which use native or modified ecdysteroid receptors expressed from transgenic constructs together with ecdysteroid agonists/antagonists as elicitors. Since this approach could involve the application of significant amounts of ecdysteroids to mammals, it is necessary to have a good understanding of the pharmacokinetics and metabolism of exogenous

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ecdysteroids in these animals. The promise of such systems derives from the tight regulation of genes placed under control of the ecdysteroid receptor and an appropriate elicitor, which are not endogenous components of mammalian systems. However, the presence of ecdysteroids in the mammalian diet (predominantly in the form of phytoecdysteroids [8]), their rapid metabolism [5,6] and the possibility that these metabolites could differentially activate the transfected genes require that the metabolism of exogenous ecdysteroids in mammals is extensively understood to avoid unexpected consequences in the use of such gene-switch systems.

Ecdysteroid metabolism has already been investigated in mammals, but these studies led to conflicting reports: Girault et al. [18] and Lafont et al. [19] analyzed the fate of injected ecdysone (E) in mice, and they identified by NMR a series of metabolites resulting from (1) 14-dehydroxylation, (2) complete reduction of the 7-en-6-one chromophore and (3) epimerization at C-3. More recent studies were performed on ingested 20E in rats [20] and humans [14], where the authors subsequently analyzed ecdysteroids in urine. Ramazanov et al. [20] administered 20E to rats and they isolated unchanged 20E together with three metabolites, which were analyzed by IR and mass spectrometry, and concluded that there was a loss of the 6-keto group followed by the reduction of the 7-ene bond. Tsitsimpikou et al. [14] analyzed the urine of a human volunteer having ingested 20 mg of "Ecdysten™" (a commercial preparation containing 20E); they collected urine and analyzed ecdysteroids by GC–MS after derivatization. They found, together with 20E, two less hydroxylated metabolites, which they tentatively identified as 2-deoxy-20-hydroxyecdysone (2d20E) and 2-deoxyecdysone (2dE) by comparison with available reference molecules. However, mass spectrometry and IR spectrometry are not sufficient to fully assess the chemical structures of these metabolites. More recently, Brandt [21] identified 14d20E in the urine of a man having ingested 20 mg of 20E, and similarly Destrez et al. [16,17] identified 14d20E and two additional metabolites (20,26-dihydroxyecdysone and 14-deoxy-20,26-dihydroxyecdysone) in the urine of calves having received 20E per os. All these data clearly show that 20E undergoes extensive modifications in mammals/humans.

In order to improve our knowledge of 20E metabolism in mammals, it was clear that only the use of a labelled molecule would allow detection of the whole spectrum of metabolites. Moreover, we suspected from previous studies [18,19] that some side-chain cleavage between carbons 20 and 22 was taking place in mice, as when we injected 20E tritiated at positions 23 and 24, a large proportion of the radioactivity became volatile [Lafont and Girault, unpublished observations]. For that reason, it was decided to prepare a radiolabelled molecule tritiated on the steroid nucleus (in the stable positions $1\alpha/2\alpha$) and then to analyze its metabolism after administration to mice.

2. Materials and methods

2.1. Animals

Five-week old Swiss mice weighing 30–35 g were used for the experiments. They had free access to water and were fed with a standard chow diet. Intraperitoneal injections of 20-hydroxyecdysone were performed in 9‰ saline. The different experiments were performed with sets of 3 mice placed in metabolic cages allowing the collection of mixed urine and faeces in a 300-mL beaker filled with ethanol in order to minimize subsequent bacterial metabolism. As our experiments were essentially qualitative, we did not attempt to collect urine separately as we knew from earlier studies that urine represents a very minor route of ecdysteroid excretion in mice [18,19].

2.2. Synthesis of reference ecdysteroids

Ultra-pure 20E (99.7%) was a generous gift from Dr. Juraj Harmatha (Prague, Czech Republic). 14-Deoxy-20-hydroxyecdysone (14d20E) was initially prepared from 20E by phototransformation [22] and later according with the procedure of Zhu et al. [23]. Poststerone and 14-deoxypoststerone were prepared from 20E and 14d20E using a side-chain cleavage method described by Petersen et al. [24].

2.2.1. Synthesis of poststerone

Side-chain cleavage of 20E was achieved according to the method described by Petersen et al. [24]. In a typical procedure, ecdysteroid (42 μmol, 20 mg) was dissolved in pyridine (1.5 mL; Aldrich > 99%) and pyridinium chlorochromate (30 mg) was added. The reaction was left for 3 h at room temperature and then water (3 mL) was added to stop the reaction. Monitoring by HPTLC indicated efficient conversion (almost no starting ecdysteroid remaining). The reaction mixture was diluted to 20 mL with water and purified by SPE, yielding poststerone (10 mg).

2.2.2. Synthesis of 14-deoxyecdysteroids

14-Deoxyecdysteroids were generated from the parent ecdysteroid (20E or poststerone) by the procedure of Zhu et al. [23]. The ecdysteroid (20E, 420 μmol) was dissolved in 2.4 mL glacial acetic acid in a Reacti-vial and 256 mg Zn powder added. The reaction was stirred over-night (16–24 h) at 67 °C. The reaction was monitored by HPTLC indicated the presence of one major UV-absorbing band in addition to some remaining parent ecdysteroid. The reaction mixture was filtered through a sintered glass funnel and washed with EtOH (4 mL), rotary evaporated, adsorbed on to Celite (2 g) and then applied to a SiO_2 column (20 g in CH_2Cl_2) and eluted with 200 mL each of 0, 5, 10 and 15% MeOH in CH_2Cl_2, collected as 20 mL fractions. The fractions containing the putative 14-deoxyecdysteroids were combined (323 μmol), dissolved in ca. 3 mL dichloromethane–isopropanol–water (125:30:1.5 v/v/v) and separated on a Zorbax Sil semi-preparative column (250 mm × 9.6 mm; 5 μm), eluted at 4 ml/min and monitored at 270 nm (to reduce sensitivity). This separated the 14α-H and 14β-H epimers, which were collected separately: [14α-H]14d20E (retention time 12.0 min, 90 mg), and [14 β-H]14d20E (retention time 13.5 min, 40 mg). A similar protocol was used with poststerone to prepare [14α-H]deoxypoststerone and [14β-H]deoxypoststerone.

By appropriate combination of the above synthetic steps, the following reference ecdysteroids were prepared: [14α-H]14-deoxy-20-hydroxyecdysone, [14β-H]14-deoxy-20-hydroxyecdysone, poststerone, [14α-H]14-deoxypoststerone, [14β-H]14-deoxypoststerone. All these products were fully characterised by NMR and MS.

2.3. Preparation of [1α,2α-$^3H_2$]20E

Initially, a 20-hydroxyecdysone derivative bearing a 1,2-unsaturation was prepared [25]. Tritiation of this compound was performed by Moravek Biochemicals Inc. (Brea, CA92821, USA) using 10% Pd/C as catalyst with tritium gas (EtOH solution, 1 h, ratio of compound/catalyst 3:1) and yielded 50 mCi [1α,2α-^{3}H]2-deoxy-20-hydroxyecdysone (spec. act.: 1.7 TBq/mmol [46 Ci/mmol]; radiochemical purity: 99.7%). The latter was stored in ethanol (1 mCi/mL) under argon at −25 °C.

One mCi was then converted into 20E by using *Drosophila* S2 cells transfected with an expression construct for an ecdysteroid 2-hydroxylase (*Drosophila* gene *sad* or CYP315A1; [26]). *Sad* cDNA was cloned in the pBRAcpA expression vector under the control of the actin 5C promoter and transfected into S2 cells (2.2×10^7 cells) using DDAB-mediated transfection [26]. Three days

after transfection, the medium (Schneider's medium + 5% FCS) was replaced by fresh medium (11.0 mL per vial) and the radioactive 2d20E was added in a small volume of ethanol (74 MBq per vial, about 1 μg/mL). After 3 days' incubation at 25 °C, media and cells were collected. After dissociation in an ultrasonic bath, samples were centrifugated and the aqueous supernatants were processed through preconditioned Sep-Pak C_{18} (0.5 g) cartridges [27]. Ecdysteroids were eluted with 5 mL ethanol. After evaporation of the solvent, they were separated by low-pressure liquid chromatography on a silica phase (Merck 60, 10 g) using chloroform: methanol (85:15, v/v) as eluent. The radioactivity present in collected fractions (2 mL each) was measured in a liquid scintillation counter (Kontron Betamatic IV), and the fractions of interest (fractions 24–28) were pooled, dried, and then kept in ethanol at −20 °C until use. High purity [^{3}H]20E was thus obtained (purity ≥ 98%), as verified by reversed-phase HPLC of an aliquot. The rate of transformation of 2d20E into 20E varied beetween 32 and 50%, depending on the experiment.

2.4. High-performance thin-layer chromatography (HPTLC)

The progress of chemical reactions was monitored by HPTLC on Merck GF_{254} aluminium-backed HPTLC plates, using chloroform/ethanol (9:1, v/v or 4:1, v/v) for development. UV-absorbing ecdysteroids were detected by fluorescence quenching under short-wavelength light and then plates were sprayed with 5% (v/v) sulphuric acid in ethanol, followed by heating at 80 °C for 10 min, to detect non-UV-absorbing compounds by charring.

2.5. Solid-phase extraction (SPE) for sample clean-up

Sep-Pak C_{18} cartridges (Millipore; 0.5 g) were activated with methanol (5 mL) and water (10 mL). Samples were diluted with water and applied to the column and the flow-through collected. Ecdysteroids were then eluted with methanol (5 mL). When not all the ecdysteroids were retained on the cartridge, the column was re-activated and the flow-through passed through again. The methanol fractions were then combined.

2.6. Extraction of urine + faeces

Faeces (and urine) were homogenized with an Ultra-Turrax™. The suspension was filtered over Celite (5 g) and the clear supernatant was evaporated to dryness, then redissolved in 50 mL MeOH. An insoluble oily residue remained and was redissolved in 50 mL EtOH, then discarded as it did not contain any detectable radioactivity. The MeOH phase was evaporated and the residue was taken up in 7 mL MeOH, 10 mL hexane and 3 mL water. The hexane epiphase was removed and replaced with a further 3 mL hexane. The combined hexane phases did not contain any radioactivity and were discarded. The aqueous MeOH solution was evaporated and the residue was redissolved in *ca.* 5 mL of MeOH and stored at 4 °C in a glass tube until use.

Large-scale experiment included firstly a partition between water and chloroform (100 mL each), then the chloroform phase was re-extracted with water (70 mL); finally hexane (270 mL) was added to allow the less polar metabolites to partition in the water phase. The aqueous phases were combined (200 mL) and adsorbed on a Sep-Pak Vac C_{18} cartridge (10 g) and the cartridge was then eluted sequentially with 200 mL each of 20%, 50%, 75% and 100% aq. MeOH. The 50% and 75% fractions contained >94% of the total radioactivity and were taken together, evaporated to dryness and then dissolved in 1 mL HPLC solvent for further purification by preparative HPLC (see below).

2.7. HPLC analyses

For analytical HPLC, one reversed-phase and one normal-phase HPLC systems were used: System 1: Spherisorb 5ODS2 (Agilent Technologies) (25 cm long, 4.6 mm i.d.), eluted with a linear gradient (in 30 min) 10–40% B (acetonitrile–isopropanol, 5:2) in water containing 0.1% trifluoroacetic acid, then (for 5 min) 100% B, flow-rate 1 ml/min; System 2: Zorbax-SIL (DuPont) (25 cm long, 4.6 mm i.d.), solvent dichloromethane–isopropanol–water (DIW) (125:25:2), 1 ml/min.

Preparative normal-phase HPLC was used to purify metabolites in large-scale experiments using a Zorbax-SIL column (DuPont) (250 mm long × 9.4 mm i.d.) eluted at a flow-rate 4 mL min^{-1}. Two different solvent systems were used, according to sample polarity: System 3, solvent DIW (125:25:2); System 4: solvent DIW (125:15:0.5), and 2 mL fractions (0.5 min) were collected.

2.8. Mass spectrometry

Mass spectra were recorded in desorption/chemical ionisation (CI/D) mode with ammonia as the reagent gas on a Jeol JMS-700 spectrometer.

2.9. Nuclear magnetic resonance (NMR)

NMR spectra were obtained on a Bruker Avance500 at 300 K. The samples were lyophilised and dissolved in D_2O. $TSPd_4$, 3-(trimethylsilyl) [2,2,3,3-d_4] propionic acid, sodium salt, was used as internal reference for proton and carbon shifts ($\delta \pm 0.2$ ppm). Chemical shifts are expressed in ppm. 1D ^{1}H and ^{13}C spectra and 2D COSY, TOCSY, PFG-HSQC and PFG-HMBC NMR spectra have further facilitated the ^{1}H and ^{13}C assignments [28,29].

3. Results

3.1. Analytical injection experiment with [^{3}H]-20E

Most (>85%) of the injected radioactivity was recovered in the faeces within one day after injection. HPLC analysis of excreta showed the presence of four major radioactive peaks, which corresponded to unconverted 20E and three less polar metabolites (Fig. 1), which comigrated in both RP- and NP-HPLC respectively with synthetic 14d20E, poststerone and 14d-poststerone. However, this was not considered as a sufficient evidence for the identification of these metabolites, hence the decision to inject simultaneously unlabelled 20-hydroxyecdysone in order to isolate sufficient amounts of these metabolites for their subsequent complete characterization by MS and NMR.

3.2. Preparative injection experiment

Three mice were injected daily with a mixture of [^{3}H]20E and unlabelled 20E (1 mg/mouse/day) over a 7-day period, i.e. corresponding to a total of 14 μCi and 21 mg (20E injected). From the faeces extract, we recovered approx. 75% of the injected radioactivity. RP-HPLC analysis of an aliquot of crude faeces extract (Fig. 2) showed that the pattern of metabolites was very complex with a prominent peak comigrating with 14d20E and low amounts of poststerone.

The faeces extract was processed as described in Section 2, then purified by preparative NP-HPLC (solvent System 3) giving 8 fractions A–H, the most polar of which, fraction H, corresponded to 20E (Fig. 3). The purity of each fraction was tested by RP-HPLC on an aliquot (1%) and, whenever necessary, the whole fraction was purified by one or two additional HPLC steps using System 4 and,

Table 1
Characterization of the different metabolites isolated in the preparative experiment—see chromatogram in Fig. 3 (bold characters refer to major components).

NP-HPLC peak	Fraction number (System 3)	Subfraction	Ret (min) (System 4)	Subfraction	Ret (min) (System 1)	Molecular mass (g/mol)
A	12–13	A1	14.0	A1-1	17.3	
		A2		A1-2	26.1	
			16.7	A2-1	18.4	346
				A2-2	23.6	
B	18–26	**B1**	47.0	**B1**	25.5	348
		B2	62.0	B2-1	21.9	
				B2-2	24.8	348
		B3	82.0	B3-1	16.3	
				B3-2	18.4	362
				B3-3	24.0	
				B3-4	26.3	
C	29–32			**C**	20.3	464
D	36–38	**D1**	35.0	D1-1	15.3	
				D1-2	20.2	
				D1-3	22.5	350
				D1-4	25.6	
				D1-5	30.7	
E	50–52	**E1**		E1-1	9.9	
				E1-2	12.9	
				E1-3	20.5	466
F	54–59	**F1**		**F1-1**	13.4	364
				F1-2	15.4	
				F1-3	16.4	
				F1-4	20.5	
G	66–69			G-1	11.4	
				G-2	15.3	
H	86–95	**H**		**H**	15.8	480

Table 2
^{1}H-NMR data of 20E and its three major metabolites (solvent CD_3OD, $T = 300$ K; (δHCD_2OD) = 3.31 ppm, ref. $TSPD_4$).

^{1}H	20-Hydroxyecdysone (20E)	14-Deoxy-20E (C)	Poststerone (B3-2)	14-Deoxy-poststerone (A2-2)
1-Ha	1.43	1.42	1.44 (d,d, 13.3, 11.9)	1.42
1-He	1.78	1.77	1.81	1.77
2-Ha	3.83 (d,d,d, 12, 3, 3)	3.84 (d,d,d, 12.1, 4.4, 3.3)	3.85 (d,d,d, 12, 4, 3)	3.84 (ddd, 12.1, 4.4, 3.3)
3-He	3.94 (ddd, 3,3,3)	3.94 m (q, 2.8, $w_{1/2}$ = 7)	3.96 (d,d,d, 3, 3, 3, $w_{1/2}$ = 10)	3.94 m ($w_{1/2}$ = 7)
4-Ha	1.65	1.68	1.71	1.68
4-He	1.75	1.72	1.74	1.71
5-H	2.38 (d,d, 12, 5)	2.38 (d,d, 12.1, 5.1)	2.39 (d,d, 12.6, 4.8)	2.38 (m)
7-H	5.80 (d, 2.5)	5.63 (t, br, 2.4)	5.81 (d, 2.5)	5.65 (tbr, 2.1)
9-Ha	3.14 (m,$w_{1/2}$ = 22)	2.67 (m,br, $w_{1/2}$ = 23)	3.19 (m, $w_{1/2}$ = 23)	2.73 (m,br,$w_{1/2}$ = 21)
11-Ha	1.65	1.76	1.68	1.75
11-He	1.78	1.90	1.89	1.98
12-Ha	2.13 (t,d, 13, 5)	1.59	2.33 (t,d, 12.9, 4.8)	1.77
12-He	1.85	2.29 (d,d,d, 12.6, 4.2, 2.8)	1.82	2.22
14-H	–	2.22 (d,d,br 11.6, 4.6)	–	2.38 (m)
15-Hβ	2.00	1.69	1.99	1.78
15-Hα	1.55	1.59	1.69	1.67
16-Hα*	1.95	1.98	2.24	2.21
16-Hβ*	1.75	1.69	1.89	1.84
17-H	2.39 (m)	1.83	3.33 (d,d,d, 9.3, 7.9, 1)	2.87 (t, 9.2)
22-Hb	3. 33 (d,d, 11, 1.7)	3. 33 (d,d, 11, 1.7)	–	–
23-Ha	1.30	1.28	–	–
23-Hb	1.65	1.61		–
24-Ha	1.75	1.79	–	–
24-Hb	1.45	1.42		–
18-Me	0.89 (s)	0.83 (s)	0.62 (s)	0.58 (s)
19-Me	0.96(s)	0.97 (s)	0.96 (s)	0.97 (s)
21-Me	1.19 (s)	1.23 (s)	2.15 (s)	2.17 (s)
26-Me	1.19 (s)	1.19 (s)	–	–
27-Me	1.20 (s)	1.21 (s)	–	–

Multiplicity of signals: *s* – singlet; *d* – doublet; *t* – triplet; *m* – multiplet. *br* – broad signal. $w_{1/2}$: width at half-height in Hertz; δ in ppm; *assignments could be reversed. t* deceptively simple triplet (4-Ha and 4-He isochronous).

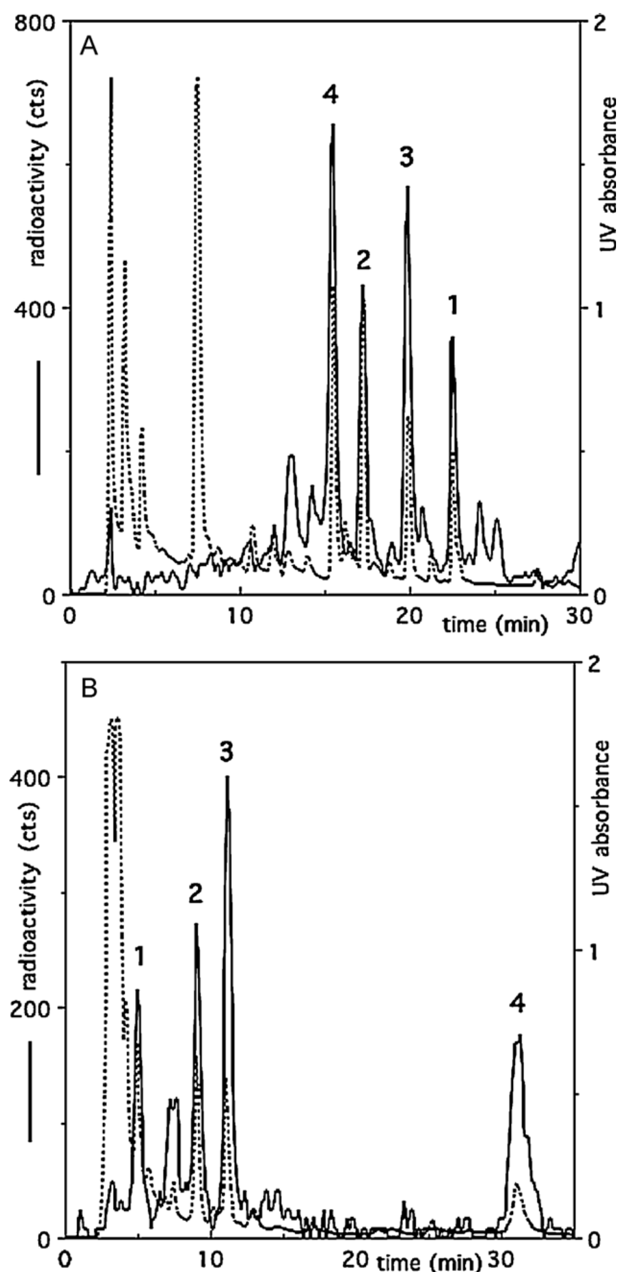

Fig. 1. HPLC analysis of mice excreta by HPLC. The dotted lines correspond to UV absorbance (254 nm) of the extract spiked with the four reference compounds. Reference ecdysteroids are (1) 14-deoxypoststerone, (2) poststerone, (3) 14-deoxy-20-hydroxyecdysone and (4) 20-hydroxyecdysone. HPLC conditions: (A) RP-HPLC (System 1) and (B) NP-HPLC (System 2).

finally, System 1. The data are reported in Table 1, together with the molecular masses of the major subfractions.

3.3. Identification of major metabolites A2-2, B3-2 and C

The major metabolites were fully identified by MS and NMR (Tables 2 and 3) and comparison with the different reference ecdysteroids.

Metabolite A2-2 was identified as 14-deoxypoststerone by comparison of its chromatographic behaviour, mass spectrum and ^{1}H NMR spectrum in CD_3OD with an authentic reference sample. The mass spectrum gives major ions at m/z 364 $[MH+NH_3]^+$ and 347 $[MH]^+$ consistent with a molecular mass of 346 g/mol (i.e. poststerone – 16 a.m.u.). The NMR spectrum of metabolite A2-2 differs

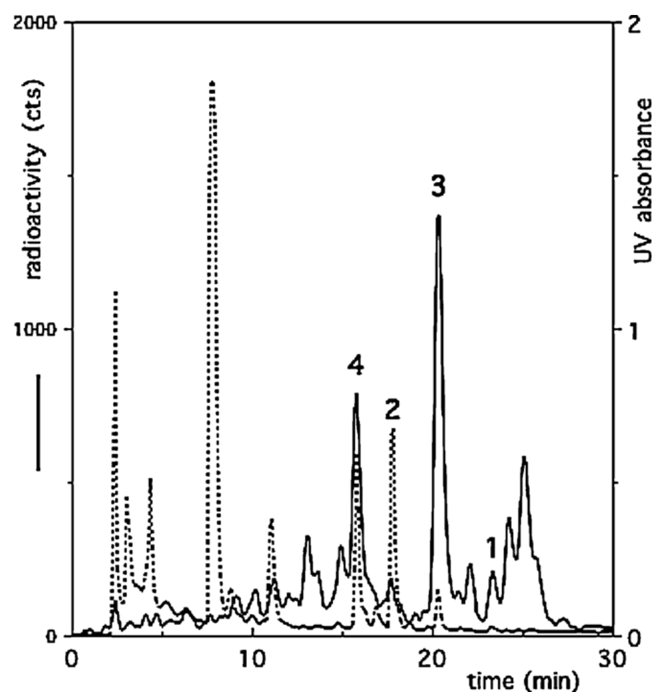

Fig. 2. RP-HPLC separation of 20E metabolites obtained after daily injections of [^{3}H]20E + unlabelled 20E over 7 days. Same conditions as for Fig. 1A. The dotted line corresponds to UV absorbance (254 nm) of the extract spiked with three reference compounds, 20-hydroxyecdysone (4), poststerone (2) and 14-deoxy-20-hydroxyecdysone (3).

from that of poststerone with regard to the following points: the presence of a 14-H signal at δ 2.38 ppm, the disappearance of the 14α-OH that leads to low frequency (upfield) shift of the assigned signal for 9-H ($\Delta\delta = -0.44$ ppm), and a small low frequency shift of the 7-H signal.

Metabolite B3-2 was identified as poststerone by its chromatographic behaviour and by comparison of its ^{1}H and ^{13}C NMR spectra in CD_3OD with an authentic reference sample.

Metabolite C was identified as 14-deoxy-20-hydroxyecdysone by comparison of its chromatographic behaviour, mass spectrum,

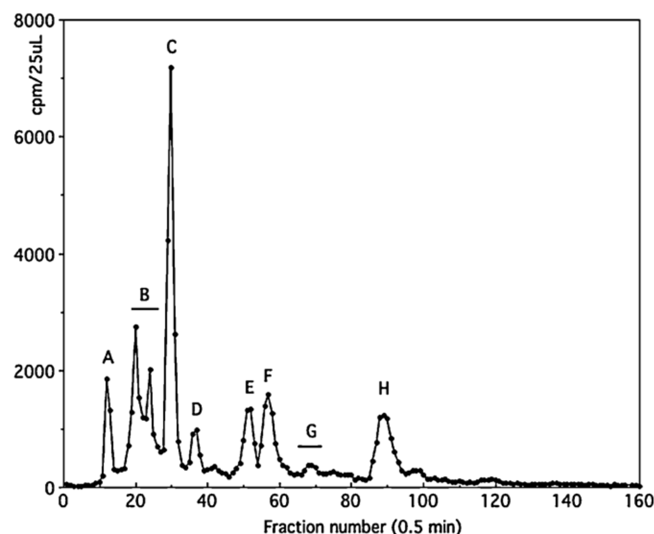

Fig. 3. Preparative NP-HPLC of mouse metabolites (System 3). Compound H corresponds to unmetabolized 20E.

Table 3
^{13}C-NMR data of 20E and its three major metabolites (solvent CD_3OD, $T = 300$ K, δ ($^{13}CD_3OD$) = 49.0 ppm).

^{13}C	20-Hydroxy ecdysone	14-deoxy20E	Poststerone	14-deoxy poststerone
C-1	37.5	37.0	37.2	37.2
C-2	68.7	68.3	68.5	68.4
C-3	68.5	68.2	68.2	68.3
C-4	32.8	32.8	32.7	32.9
C-5	51.8	51.4	51.5	51.5
C-6	206.4	205.6	205.9	205.6
C-7	122.1	122.0	122.3	122.4
C-8	167.9	168.3	166.3	166.9
C-9	35.1	38.9	34.9	38.9
C-10	39.3	38.9	39.1	38.9
C-11	21.5	22.6	21.4	22.6
C-12	32.5	40.3	30.9	38.7
C-13	48.7	46.7	48.8	46.7
C-14	85.3	56.7	84.8	56.2
C-15	31.8	23.2	31.9	23.6
C-16	21.5	22.4	22.1	23.4
C-17	50.5	55.9	59.9	63.8
C-18	18.0	14.4	17.3	13.8
C-19	24.4	24.4	24.2	24.4
C-20	77.9	77.4	212.2	211.3
C-21	21.1	20.7	31.3	31.3
C-22	78.4	77.8		
C-23	27.4	26.9		
C-24	42.4	42.0		
C-25	71.3	71.1		
C-26	29.1	28.7		
C-27	29.6	29.6		

and ^{1}H NMR spectrum in CD_3OD with an authentic reference sample. Mass spectrometry indicates a molecular weight of 464, corresponding to $C_{27}H_{44}O_6$ (loss of one oxygen). The NMR spectrum of metabolite C differs from that of 20-hydroxyecdysone with regard to the presence of a 14-H signal at δ 2.22 ppm. The disappearance of the 14α-OH leads to low frequency shifts of the signals for 9-H ($\Delta\delta = -0.47$ ppm), 12-Hax ($\Delta\delta = -0.54$ ppm), 17-H ($\Delta\delta = -0.56$ ppm), and 15-Hb ($\Delta\delta = -0.31$ ppm).

Metabolite H was identified as unconverted 20-hydroxyecdysone.

3.4. Partial characterization of minor metabolites

Some other metabolites were obtained in amounts sufficient for a partial characterization:

Metabolite B1: mass spectrum (CI/D) gives major ions at m/z 366 $[MH+NH_3]^+$ and 349 $[MH]^+$ indicating a MM of 348 (=346 + 2) a.m.u.
Metabolite B2-2: mass spectrum (CI/D) gives major ions at m/z 366 $[MH+NH_3]^+$, 349 $[MH]^+$ and 331 $[MH-H_2O]^+$ indicating a MM of 348 (=346+2) a.m.u.
Metabolite D1-4: mass spectrum (CI/D) gives major ions at m/z 368 $[MH+NH_3]^+$ and 333 $[MH-H_2O]^+$ indicating a MM of 350 (=346 + 4) a.m.u.
Metabolite F1-1: mass spectrum (CI/D) gives major ions at m/z 382 $[MH+NH_3]^+$, 365 $[MH]^+$, 347 $[MH-H_2O]^+$ and 329 $[MH-2H_2O]^+$ indicating a MM of 364 (=362 + 2) a.m.u.

These metabolites seem to represent various reduced derivatives of poststerone or 14-deoxypoststerone. They no longer absorb UV-light at 245 nm, thus providing evidence that the chromophore of ring-B was modified (by the reduction of the 6-one, or of the 7,8-ene). The possibility of a reduction of the 20-one has also to be considered (e.g. for metabolite D1-4). The identification of these metabolites is presently under investigation (by comparison with an additional set of synthetic reference compounds).

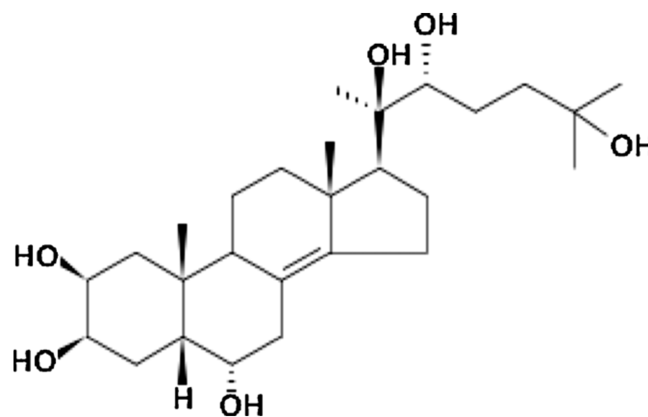

Fig. 4. Structure of metabolite E1-3.

3.5. Identification of metabolite E1-3

Metabolite E1-3 was identified as 2β,3β,6α,22*R*,25-pentahydroxy-5βcholest-8,14-ene (Fig. 4) based on the following evidence: (1) mass spectroscopy indicates a molecular weight of 466, i.e. that of 14d20E + 2 a.m.u (480–16 + 2), (2) the compound no longer absorbs UV-light (which indicates a modification of the chromophore on ring-B), and (3) NMR analyses – ^{1}H 1D and 2D experiments 2D COSY, TOCSY, NOESY and 2D ^{1}H-^{13}C correlations. HSQC and HMBC have allowed a complete structure elucidation (Table 4). The following major differences between the spectra of metabolite E1-3 and 20E were noted. There was disappearance in the ethylenic area of the 7-H signal. A new signal appears in the >CHOH region at $\delta = 4.05$ ppm, 1H. This proton is coupled to three protons at $\delta = 2.42$, 1.82 and 1.88 ppm, respectively, assigned by 2D experiments to 7-Heq, 7-Hax and 5-HβI. Its width at half-height ($w_{1/2} \approx 30$ Hz) is in agreement with an axial position which is confirmed by a 2D NOESY experiment, where one can observe a nOE correlation between 19-Me and this proton. This signal can be assigned to the 6-Hβ_{ax}. Examination of methyl signal correlations in 2D HMBC shows that methyl signals at $\delta = 1.00$ (s) and 1.21 (s) ppm share a correlation with the same ^{13}C signal assigned thanks to HSQC to C-17, then these two methyl signals can be assigned, respectively, to 18-Me and 21-Me. Consequently, the methyl signal at $\delta = 0.84$ (s) ppm is assigned to 19-Me and $\delta = 1.17$ (s), $\delta = 1.19$ (s), to 26-Me and 27-Me. These assignments are in perfect agreement with the ^{13}C chemical shift observed from HSQC of the five methyl signals of this molecule. The 18-Me methyl signal presents 4 correlations in 2D HMBC: C-17 ($\delta = 57.8$ ppm, see above), C-12 ($\delta = 40.1$ ppm), C-13 ($\delta = 46.1$ ppm) and one with a quaternary ethylenic carbon at $\delta = 147.1$ ppm, which could be assigned unambiguously to C-14, thus establishing the presence of an ethylenic bond between C-14 and C-8. This is in agreement with the chemical shifts observed for the proton signals of 7-H_{eq},7-H_{ax}, 9-H and 15-H_a, 15-H_b which were found as expected in the zone of allylic protons. On the other hand, the side-chain of metabolite E1-3 does not present any major difference with respect to 20-hydroxyecdysone. The signals of 2-Hax ($w_{1/2} \approx 24$ Hz) and 3-Heq ($w_{1/2} \approx 12$ Hz) are in agreement with, respectively, axial and equatorial protons. The 5β-H stereochemistry of the junction between ring-A and ring-B is established thanks to the observation of a strong NOESY correlation between protons of the alpha-side (2-H_{ax} and 9-H_{ax}), as observed for 20-hydroxyecdysone.

Table 4
Chemical shifts of metabolite E1-3 in H_2O/D_2O at 300 K.

^{1}H	20E D_2O	E1-3 H_2O/D_2O	^{13}C	Multiplicity	E1-3 H_2O/D_2O
1-Ha	1.38 (t,13)	1.43	C-1	CH_2	39.1
1-He	1.88	1.67			
2-Ha	3.99 (m,$w_{1/2}$ = 22)	3.84 (m,$w_{1/2}$ = 24)	C-2	CH	70.0
3-He	4.07 (m,$w_{1/2}$ = 8)	4.09 (m,$w_{1/2}$ = 12)	C-3	CH	71.4
4-Ha	1.75	1.78[b]	C-4	CH_2	28.2
4-He	1.75	1.82[b]			
5-H	2.36 t[b]	1.88	C-5	CH	44.2
6-H	–	4.05 (m,w1/2 = 30)	C-6	CH	70.3
7-H	5.97 (d, 2.5)	7-Ha 1.82 7-He 2.42 (dd, 13.8, 5.5)	C-7	CH_2	35.0
			C-8	C	[a]
9-Ha	3.11 (m, $w_{1/2}$ = 22)	2.26	C-9	CH	37.8
			C-10	C	41.4
11-Ha	1.73	1.51[b]	C-11	CH_2	22.3
11-He	1.86	1.61[b]			
12-Ha	1.95	1.24	C-12	CH_2	40.1
12-He	1.95	2.14			
			C-13	C	46.1
			C-14	C	147.1
15-Hβ	2.05	2.25	C-15	CH_2	27.8
15-Hα	1.65	2.32			
16-Hα[b]	1.90	1.62	C-16	CH_2	23.7
16-Hβ[b]	1.80	1.83			
17-H	2.34 (m)	1.51	C-17	CH	57.8
18-Me	0.87 (s)	1.00 (s)	C-18	CH_3	21.9
19-Me	1.00 (s)	0.84 (s)	C-19	CH_3	26.2
20-H	–	–	C-20	C	80.3
21-Me	1.24 (s)	1.21 (s)	C-21	CH_3	22.0
22-Hb	3.43 (d, 10)	3.47 (d, 11.6)	C-22	CH	79.8
23-Ha	1.33	1.26	C-23	CH_2	28.6
23-Hb	1.65	1.53			
24-Ha	1.80	1.45	C-24	CH_2	43.1
24-Hb	1.51 (d,t, 12.8, 3.4)	1.73			
			C-25	C	74.3
26-Me	1.23 (s)	1.17 (s)	C-26	CH_3	29.9
27-Me	1.24 (s)	1.19 (s)	C-27	CH_3	30.9

[a] Not detected (too low concentration).
[b] Assignments could be reversed.

4. Discussion

4.1. Ecdysteroids undergo extensive metabolic conversion in mammals

A rapid metabolism (=a short half-life) of either injected or ingested ecdysteroids has already been documented by several authors [30–33]. Thus, Dzukharova et al. [32] reported a plasmatic half-life of 8.15 min of injected 20E [50 mg/kg] in mice; Simon and Koolman [1] found a half-life for ingested 20E of 4.1 h (20E [0.2 mg/kg] was ingested by a human volunteer and ecdysteroid concentration was measured by RIA in serum samples). Using radiolabelled 20E, Hikino et al. [30,31] showed that faeces were the major excretion route in mice (urine being a minor one). Major faecal excretion was also observed in mice injected with ecdysone [18,19].

The metabolic fate of 20-hydroxyecdysone in mammals is still a matter of debate: Ramazanov et al. [20] described metabolites of 20E in rat urine lacking the 6-oxo group and where the 7-ene was reduced. Tsitsimpikou et al. [14] described dehydroxylated metabolites of 20E in humans (the removed –OH were tentatively assigned to positions 2 and 20). On the other hand, metabolites of ecdysone in mice were mainly 14-dehydroxylated and then reduced on ring-B [18,19]. In the present study, 14-dehydroxylation of 20E was unambiguously demonstrated as a major pathway. This result is consistent with the data of Brandt [21], who identified 14-deoxy-20E in human urine following 20E ingestion, and Destrez et al. [16,17], who identified both 14-deoxy- and 26-hydroxy-metabolites in the urine of calves having ingested 20E. 14-Deoxy-20E was also previously observed in the faeces of the insect *Gryllus bimaculatus* [34]. Such a dehydroxylation reaction is most probably caused by gut bacteria, already known to perform dehydroxylation at positions 16 and 21 in steroids and at position 7 in bile acids [35,36].

A second major reaction not previously reported concerns side-chain cleavage between carbons 20 and 22. Indeed, 20E possesses a 20,22-diol, which is reminiscent of that formed during the multistep process of side-chain cleavage of cholesterol to give pregnenolone and catalyzed by a cytochrome P450 enzyme (CYP11A1). Whether this vertebrate enzyme could accept 20E as a substrate remains however to be demonstrated. Alternatively, this reaction could result from the action of some gut microorganisms. On the other hand, we did not detect further side-chain cleavage between carbons 17 and 20, as that is known during the biosynthesis of vertebrate sex hormones: such a reaction would have led to the formation of rubrosterone (and 14-deoxy-rubrosterone). Rubrosterone is present in some plants [37] and it has already been described as a metabolite of 20E incubated with some microorganisms [38], so its presence or that of any derivative (e.g. 14-deoxy- and/or some reduced form) has to be further investigated.

The reduction reactions may probably take place in liver, which contains a set of enzymes (20α- and 20β-HSDs, 3α-HSD, 5α- and 5β-reductases) classically involved in steroid hormone catabolism [39], but once again gut bacteria could possibly perform similar reactions [36], thus making difficult to conclude, unless metabolic studies with gut bacteria are performed. Following reduction of the 6-ketone to a 6α-OH group, it appears that in the absence of a 14-OH group, the 7(8)-ene can migrate to a more stable 8(14)-position,

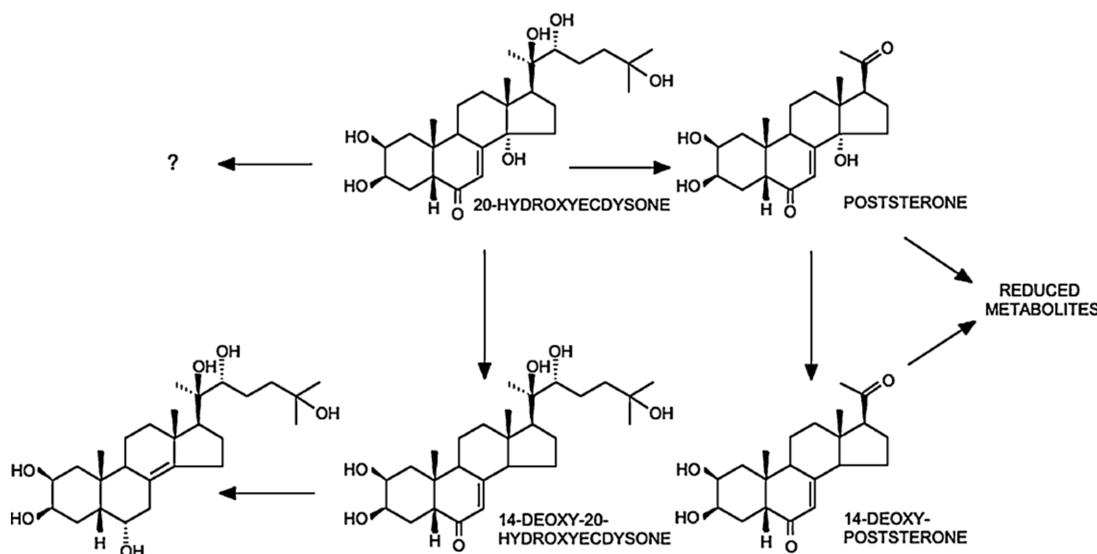

Fig. 5. Metabolic pathways of injected 20-hydroxyecdysone in mice. It is proposed that 14-dehydroxylation is performed by gut bacteria, and that the various reductions result from an enterohepatic cycle and are mainly performed in mouse liver, although a contribution by the gut flora cannot be excluded.

as evidenced by the isolation of metabolite E1-3. Interestingly, a related compound (14-deoxy,8(14)-ene, but retaining the 6-one) was formed upon UV-irradiation of 20E [40]. Whether reduction of the 6-one requires previous removal of the 14-OH remains also to be investigated, in order to ascertain the sequence of catabolic reactions. Among minor metabolites, we expect to find other 6α-OH-7-ene ecdysteroids or even compounds with a fully reduced ring-B, as we found after injecting ecdysone [19], but this will require additional experiments. When comparing the pattern of metabolites between analytical (Fig. 1A) and preparative experiments (Fig. 2), we are led to the conclusion that the second ones contain more reduced metabolites, which could be the result of inducible activities in the liver [41]. The identified metabolic pathways are summarized in Fig. 5.

4.2. Possible relations of this metabolism to the pharmacological effects of 20E

Of course, the evidence for this complex metabolism of injected 20E raises an immediate question; which metabolites might be responsible for the pharmacological activities of 20E. Certainly, 20E itself may have direct effects: see e.g. its rapid neuromodulatory effect on the $GABA_A$ receptor [42], or the stimulation of protein synthesis in myocytes *in vitro* [13], although in the latter case no metabolic study was performed. There are no reported pharmacological effects of poststerone on mammals, but on the other hand rubrosterone was reported to be as active as 20E for stimulating protein synthesis in mouse liver [12]. Presently no experiments have been performed with 14-deoxyecdysteroids, so the potential bioactivity of such compounds remains open at the moment. Thus, when considering the possible binding of ecdysteroids to vertebrate nuclear receptors, it would be advisable to test not only 20E but also all its metabolites.

We spontaneously focus on the steroid nucleus to search for biological effects, but it might well be that the side-chain cleavage product itself (γ-hydroxy-γ-methylpentanoate) has (also) some biological activity. Such a molecule indeed represents a direct homologue of β-hydroxy-β-methylbutyrate (HMB), a leucine derivative, which has been shown to possess significant anabolic properties [43–45].

Finally, we should also consider indirect effects through the induction of steroid catabolizing activities [41,46], which might influence endogenous steroid levels, e.g. induce a decrease of cortisol levels.

Our metabolic studies may provide some assistance to laboratories concerned by doping control. At the moment ecdysteroids are not considered illegal doping substances (for increasing sporting performances or increasing the growth of farm animals), but they have already led to some preliminary investigations for such a purpose [14,15]. Thus, metabolites unique to mammalian systems (i.e. which are not natural phytoecdysteroids) could form the basis for a test for the consumption of large amounts of ecdysteroids [16,17]. Levels of ecdysteroids in a normal western diet are low (usually in the range of less than 1 mg/day [47]), unless eating large amounts of spinach or quinoa [48], whereas doses 'recommended" for bodybuilders are in the range of 100–1000 mg/day [4,5,7].

Finally, this evidence for complex metabolism together with a wide array of articles describing pharmacological effects of ecdysteroids on mammals/humans would justify further investigations of the mechanisms involved before developing ecdysteroid-inducible gene-switch systems for gene therapy in humans [6]. Although the ecdysteroid metabolites identified here have low affinities for ecdysteroid receptors in insect systems [49], the altered affinity and specificity of ecdysteroid receptors when expressed in mammalian systems [49] means that these metabolites should be assessed in mammalian cell-based gene switch systems to determine if they possess activity at concentrations which might occur after using 20E as an elicitor.

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