 doi:10.20944/preprints202105.0106.v1

Article

Urinary Elimination of Ecdysterone and its Metabolites following a Single-Dose Administration in Humans

Gabriella Ambrosio[1], Tasha Yuliandra[1], Bernhard Wuest[2], Monica Mazzarino[3], Xavier de la Torre[3], Francesco Botrè[3,4], Patrick Diel[5], Eduard Isenmann[5], Maria Kristina Parr[1,*]

[1] Institute of Pharmacy, Pharmaceutical and Medicinal Chemistry (Pharmaceutical Analysis), Freie Universitaet Berlin, Berlin, Germany; gabriella@zedat-fu-berlin.de; tasha.y@fu-berlin.de; maria.parr@fu-berlin.de
[2] Agilent Technologies, Waldbronn, Germany; bernhard_wuest@agilent.com
[3] Laboratorio Antidoping FMSI, Rome, Italy; monica.mazzarino@gmail.com; xavier.delatorre@fmsi.it; francesco.botre@unil.ch
[4] REDs – Research and Expertise in anti-Doping sciences, ISSUL – Institute of sport sciences, University of Lausanne, Lausanne, Switzerland; francesco.botre@unil.ch
[5] Department for Molecular and Cellular Sports Medicine, Institute for Cardiovascular Research and Sports Medicine, German Sport University Cologne, Cologne, Germany; diel@dshs-koeln.de
* Correspondence: maria.parr@fu-berlin.de

Abstract: Ecdysterone is a phytosteroid widely discussed for its various pharmacological, growth-promoting and anabolic effects mediated by activation of estrogen receptor beta (ERbeta). Performance-enhancement in sports was demonstrated recently, and ecdysterone was consequently included in the Monitoring Program to detect potential patterns of misuse in sport. Only few studies on the pharmacokinetics of ecdysterone in humans have been reported so far. In this study, post-administration urines in twelve volunteers (single dose of 50 mg of ecdysterone) were analyzed using dilute-and-inject liquid chromatography-tandem mass spectrometry. Identification and quantitation of ecdysterone and of two metabolites, 14-deoxy ecdysterone and 14-deoxy poststerone was achieved. Ecdysterone was the most abundant analyte present in post-administration urines, detected for more than 2 days with a maximum concentration (C_{max}) in the 2.8-8.5 h urines (C_{max} = 4.4-30.0 μg/mL). The metabolites 14-deoxy ecdysterone and 14-deoxy poststerone were detected later reaching the maximum concentrations at 8.5-39.5 h (C_{max} = 0.1-6.0 μg/mL) and 23.3-41.3 h (C_{max} = 0.1-1.5 μg/mL), respectively. Sex-specific differences were not observed. Cumulative urinary excretion yielded average values of 18%, 2.3% and 1.5% for ecdysterone, 14-deoxy ecdysterone and 14-deoxy poststerone, respectively. Ecdysterone and 14-deoxy ecdysterone were excreted following first order kinetics with half-lives calculated with three hours, while pharmacokinetics of 14-deoxy poststerone needs further evaluation.

Keywords: ecdysterone; metabolites; excretion profile; urinary pharmacokinetics;

1. Introduction

Ecdysterone (chemical structure Fig. 1a) is a steroid hormone naturally present in plants. It is the most widely used active ecdysteroid, and its pharmacological effects have been discussed since the 1980s. Studies reported the ability of this natural steroid hormone to stimulate protein synthesis, to change carbohydrate and lipid metabolism. It has also been highlighted that ecdysterone is correlated with an increased cell immunity, and that it is also endowed with adaptogenic, anti-diabetic, hepatoprotective, and anti-tumor properties [1-3]. Moreover, growth-promoting and anabolic effects in animals and in humans have been reported [3-19]. *In vitro* and *in silico* studies have shown that the anabolic effect of ecdysterone is mediated by activation of estrogen receptor beta (ER beta) [4, 20-22]. Ecdysterone is marketed as able to increase strength and muscle mass and improve performances, without having any of the classical side effects of anabolic androgenic steroids (AAS) [3, 23, 24]. For these reasons, the use of dietary supplements containing this

 doi:10.20944/preprints202105.0106.v1

"natural" steroid may be considered very attractive for athletes aiming to maximize their performances, and it has become a topic of high interest within, but not limited to, the sport context.

Only recently (since 2020), ecdysterone has been included in the Monitoring Program of the World Anti-Doping Agency (WADA), under the section "Anabolic agents, In-and Out-of-Competition" [25], to assess potential patterns of misuse in sport. This decision has been based mainly on the results of a controlled administration trial in humans, which demonstrated its performance-enhancing effects in power training [26].

Studies on the metabolism of ecdysterone have been conducted mostly in animals and only a few refer to humans [6, 27-30]. While ecdysterone does not seem to undergo phase II metabolism [27, 31], studies concur on its biotransformation, leading to the formation of dehydroxylated metabolites. However, structure assignment and information on the excretion profiles differ in some studies. Indeed, while Tsitsimpikou et al. reported a 2-deoxy ecdysterone and deoxy ecdysone as urinary metabolites [29], Brandt reported the 14-deoxy ecdysterone [30]. In both studies, the metabolism was evaluated after an administration to a male volunteer of a dietary supplement called "Ecdysten", for a final ecdysterone content of 20 mg.

Recently, in a single dose administration study, 51.5 mg of pure ecdysterone has been administered to one healthy volunteer, and the presence of 14-deoxy ecdysterone was confirmed in the post-administration urine samples by comparison with in-house synthesized reference [31]. Parr et al. reported ecdysterone as the most abundant analyte in post-administration urines, with a wider detection window than 14-desoxy ecdysterone, which was excreted later [31]. These results are similar to the one reported by Brandt [30], while Tsitsimpikou et al. reported that ecdysterone is detected in urine mainly as the deoxy ecdysone metabolite, followed by the parent compound and the deoxy ecdysterone metabolite [29].

This study aims to investigate and provide consistent analytical information regarding the urinary excretion and pharmacokinetics of ecdysterone and its metabolites in humans. More specifically, we have followed the elimination of ecdysterone and its metabolites following the administration of a single oral dose of 50 mg in twelve volunteers.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2021 doi:10.20944/preprints202105.0106.v1

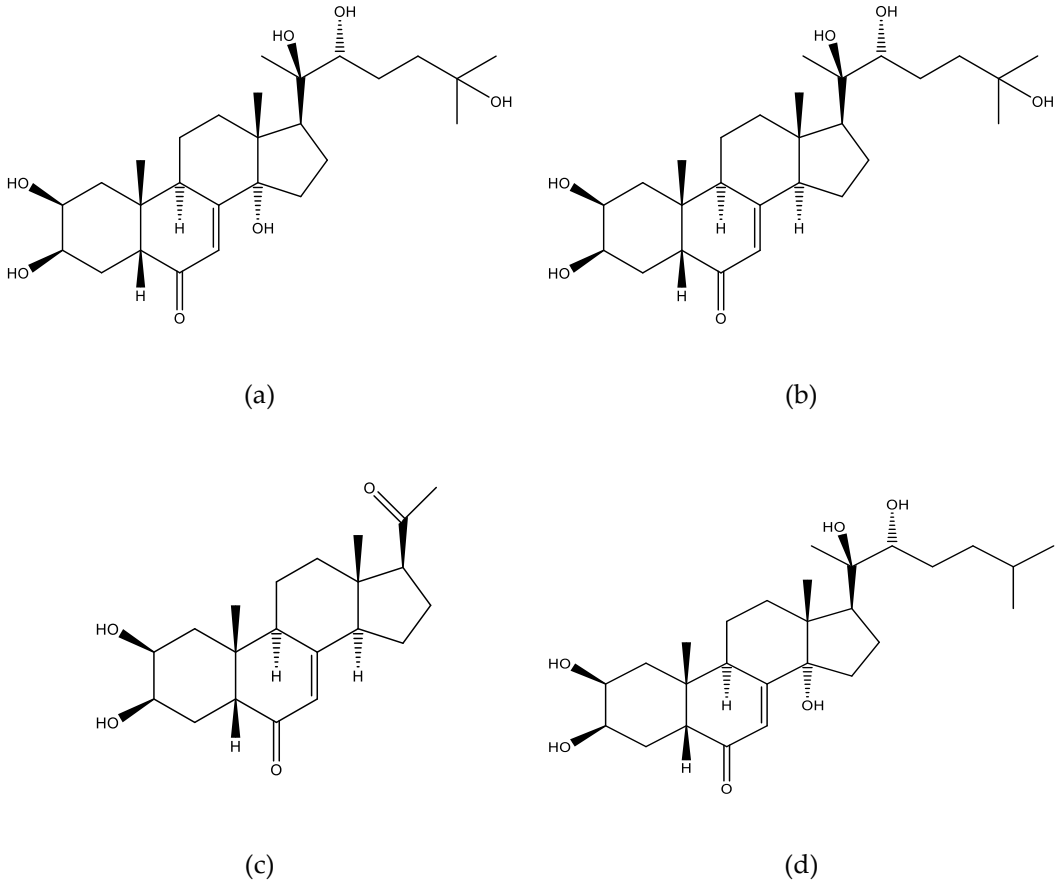


Figure 1. Chemical structure of (a) ecdysterone, of (b) 14-deoxy ecdysterone, (c) 14-deoxy poststerone and (d) ponasterone (ISTD).

2. Results

The analysis of the urines has been performed using LC-MS/MS and the mass spectrometer was operated in multiple reaction monitoring (MRM) acquisition mode, using positive ionization (ESI+). The protonated molecular ion $[M+H]^+$ for ecdysterone, was detected at *m*/*z* 481.3, for 14d-ecdysterone and ponasterone (isomers) at 465.3 *m*/*z* and for 14d-poststerone at 347.2 *m*/*z*. For the quantitation of the samples, the ion transition 481.3 →445.3 has been used for ecdysterone, 465.3→303.2 for 14d-ecdysterone, 465→447.3 for ponasterone (ISTD) and finally 347.2→329.1 for 14d-poststerone.

2.1. Validation of the analytical methodology

Prior to its application, complete validation of the analytical methodology was performed according to the European Medicines Agency (EMA) [32] and the International Council for Harmonization (ICH M10) [33] guidelines for Bioanalytical Method Validation.

The analytical procedure was validated in terms of selectivity, linearity, limit of detection (LOD) and limit of quantitation (LOQ), precision and accuracy, matrix effect, stability and carry over.

2.1.1. Selectivity

The selectivity was studied by analyzing six individual blank urines to determine if anything in the matrix interfered with the analyte(s) of interest and the internal standard (ISTD). No interfering signals at the retention time of ecdysterone (RT=2.991), 14*α*-deoxy ecdysterone (14d-ecdysterone) (RT=3.519), 14-deoxy poststerone (14d-poststerone)

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2021 doi:10.20944/preprints202105.0106.v1

(RT=4.068) and ponasterone (internal standard, ISTD) (RT=4.800) have been detected. Additionally, no interferences with other isomeric ecdysteroids, i.e. ecdysone, 14β-desoxy-ecdysterone, ponasterone, occurred due to their chromatographic resolution from the target analytes.

2.1.2. Linearity of the calibration curves, LOD and LOQ

For the response function, blank urines were spiked with ecdysterone at 12 calibration levels from 1 to 5000 ng/mL, while for 14d-ecdysterone and 14d-poststerone, at 10 calibration levels, from 1 to 1000 ng/mL. Each level of calibrants has been prepared in duplicate. Calibration curves were constructed based on the peak area ratios of the analytes to the ISTD (y-axis) versus the nominal standard concentration (x-axis).

Back calculation was performed to determine the concentrations of ecdysterone, 14d-ecdysterone and 14d-poststerone in each calibration standards, which were used for the quantitation of the analytes in quality control samples (QCs) and post-administration urines applying the equation $y= ax^2 + bx + c$, using Mass Hunter Quant Software Ver. 10 from Agilent. The weighted quadratic regression has been applied after evaluation of the linearity according to Mandel's fitting test (F-test), which resulted in a significantly better fit of the second order calibration function (quadratic) in comparison to the first order regression function (linear), with a p-value ≤ 0.05. Furthermore, testing the homogeneity of variance, according to DIN 38402 T51, a significant difference between the variances (p = 99%) has been reported. As consequence, the weighted factor 1/x has been applied. The best fit for ecdysterone was indicated by a correlation coefficient (R^2) of 0.997 while for 14d-ecdysterone R^2=0.998.

The LOD was calculated using the standard deviation (SD) of the response and the slope of calibration (LOD= 3.3*S.D./slope) [34] and corresponded to 0.24 ng/mL for ecdysterone and to 0.34 ng/mL for 14d-ecdysterone.

The LOQ was determined based on the lowest concentration in which the % error of accuracy was within ± 20% and coefficient of variation (CV) ≤ 20% [33] and corresponded to 1 ng/mL for both ecdysterone and 14d-deoxy ecdysterone.

Results are reported in Table 1.

Table 1. Calibration model, LOD and LOQ.

Analyte	Calibration model	Weighted	Calibration range (ng/mL)	R^2	LOD (ng/mL)	LOQ (ng/mL)
Ecdysterone	Quadratic	1/x	1 – 5000	0.997	0.24	1
14-deoxy ecdysterone	Quadratic	1/x	1 - 1000	0.998	0.34	1

2.1.3 Accuracy and precision

For the evaluation of intra-day accuracy, expressed as the percent of relative error (RE%) and precision, reported as the percentage of coefficient of variation (CV%), five replicates of the quality control samples (QCs) at the low concentration (LQC), at two different medium concentrations (MQC1 and MQC2) and at high concentration (HQC) were analyzed in the same day. The intermediate precision has been evaluated by injecting the LQC, MQC and HQC in three different days. The results of accuracy, intraday and intermediate precision for ecdysterone and 14d-ecdysterone were all within the acceptance values (CV% < 15%, RE<15%), indicating that the analyte and the metabolite concentration in the urine samples can be determined with reasonable precision and accuracy. Details are reported in Table 2.

Table 2. Intraday accuracy and precision and intermediate precision of ecdysterone and 14-deoxy ecdysterone.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2021 doi:10.20944/preprints202105.0106.v1

Compound	Level	Concentration (ng/mL)	Intraday (n=15)			Intermediate precision (n=15)
			Mean concentration (ng/mL) ± SD	RE (%)	CV (%)	CV (%)
Ecdysterone	LQC	1	0.92±0.11	-7.6	12.2	9.9
	1°MQC	250	248±9.9	-0.7	4.0	2.7
	2°MQC	2500	2470±70	-1.4	2.8	3.9
	HQC	5000	4770±160	-4.7	3.4	3.3
14-deoxy ecdysterone	LQC	1	1.0±0.1	-3.4	12.3	11.8
	1°MQC	50	49.7±1.2	-0.7	2.4	3.3
	2°MQC	500	507±14	1.4	2.7	3.8
	HQC	1000	949±32	-5.1	3.4	4.5

*Each value is presented as mean ± SD. CV, coefficient of variation. RE, relative error.

2.1.4 Matrix effect

For the evaluation of the matrix effect, blank urines of six different volunteers from individual donors (3 female and male) have been analyzed.

The matrix effect has been evaluated spiking ecdysterone and 14d-ecdysterone at a LQC and HQC in matrix and non-matrix samples. For each analyte and ISTD the matrix factor (MF) has been determined by calculating the ratio of the peak area in the presence of matrix to the peak area in absence of matrix (analytes and ISTD spiked in the methanol:water 10:90, v/v). The ISTD normalized MF was calculated by dividing the MF of the analytes by the MF of the ISTD. The CV of the ISTD-normalized MF calculated was lower than 15 % in all 6 lots of matrix at LQC and HQC except for 14d-ecdysterone at the LQC, in which it corresponds to 20 %. Details are reported in Table 3. These results show a high variability. Matrix-matched calibrants have been used for quantitation.

Table 3. Evaluation of matrix effects of ecdysterone and 14-deoxy ecdysterone in human urines.

Compound	Level	Concentration (ng/mL)	Matrix Effect % (mean ± SD)	CV%
Ecdysterone	LQC	1	85 ± 7	8.0
	HQC	5000	94 ± 4	4.7
14-deoxy ecdysterone	LQC	1	79 ± 16	20
	HQC	1000	90 ± 7	7.5

* Each value is presented as mean ± SD. CV, coefficient of variation.

2.1.5 Stability

The stability of ecdysterone and 14d-ecdysterone in urines was determined in triplicate using LQC and HQC samples which were analyzed immediately and again after storage as reported in Table 4. Freshly prepared QC samples (at t=0) were used as baseline to assess the stability.

To evaluate the bench-top stability, LQC and HQC samples were left at room temperature for 4, 8 and 24 hours before analysis. Freeze and thaw stability was evaluated after three cycles for LQC and HQC. Long-term stability was evaluated analyzing the LQC and HQC samples after storing them at -20°C for two weeks. The results of the stability tests applied were calculated using the peak area ratio of the analytes to the ISTD which were compared to the baseline (t=0). Comparing the mean of the ratios after a specific storage condition to the mean of the ratio at t=0, The results obtained were all within the ±15%. Details are reported in Table 4.

Table 4. Evaluation of the stability of ecdysterone and 14-deoxy ecdysterone.

	Ecdysterone	**14-deoxy ecdysterone**

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2021 doi:10.20944/preprints202105.0106.v1

		LQC	HQC	**LQC**	**HQC**
	Time/Cycle	**Stability%**	**Stability%**	**Stability%**	**Stability%**
Bench-top	0 h	100	100	100	100
	4 h	100	101	103	101
	8 h	104	98	101	100
	24 h	100	100	97	101
Long term	0	100	100	100	100
	2 weeks	94	87	91	87
Freeze-Thaw	0	100	100	100	100
	3 cycles	111	103	110	103

* Each value is presented as mean (n=3).

2.1.6 Carry over

Carry over was tested after the injection of a HQC sample. No signal higher than the 20% of the LOQ for ecdysterone and 14d-ecdysterone was detected in the blank samples (methanol). Thus, carry-over is considered irrelevant.

2.2. Post-administration urine analysis and evaluation of the urinary excretion profiles of ecdysterone and its metabolites

The elimination of ecdysterone and its metabolites in post-administration urines after a single dose administration of pure ecdysterone to 12 subjects, has been evaluated. For calibration, blank urines have been spiked with increasing concentration of ecdysterone, 14d-ecdysterone and 14d-poststerone reference standard solutions (matrix matched standard).

In this study ecdysterone has been detected in all subjects following the administration of 50 mg of pure ecdysterone. The 14d-ecdysterone and a new metabolite, the 14d-poststerone, have been detected and confirmed by comparing the retention time and the mass spectra with the reference standard material using LC-MS/MS.

The developed and validated method has enabled the quantitation of ecdysterone and 14d-ecdysterone in post-administration urines and consequently their excretion profiles have been evaluated.

Concentration data below the LOQ (but not below the LOD), have been included to evaluate the excretion profile, as their inclusion can result in a better fit of the excretion profile model, while excluding or replacing them with zero, could lead to biased pharmacokinetic parameters [35-38].

Results of the excretion profile of ecdysterone, 14d-ecdysterone and 14d-poststerone, considering the concentration versus time and the rate of excretion versus middle point time curves, are reported in Figure 2 and 3, respectively.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2021 doi:10.20944/preprints202105.0106.v1

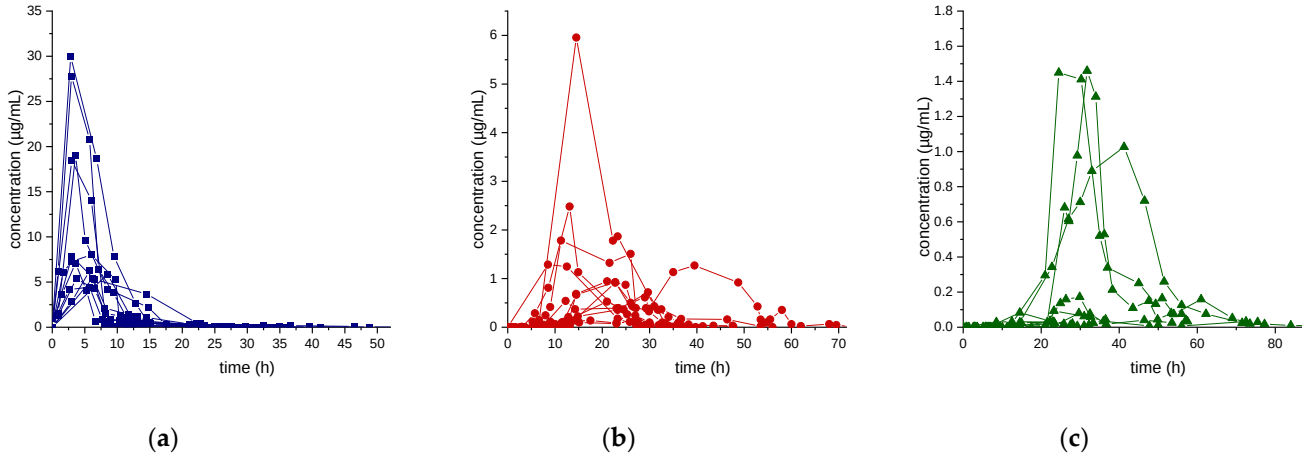


Figure 2. Urinary excretion profile (concentration-time curve) of ecdysterone (a), 14-deoxy ecdysterone (b) and 14-deoxy poststerone (c) following a single dose administration of 50 mg of pure ecdysterone in humans (n=12).

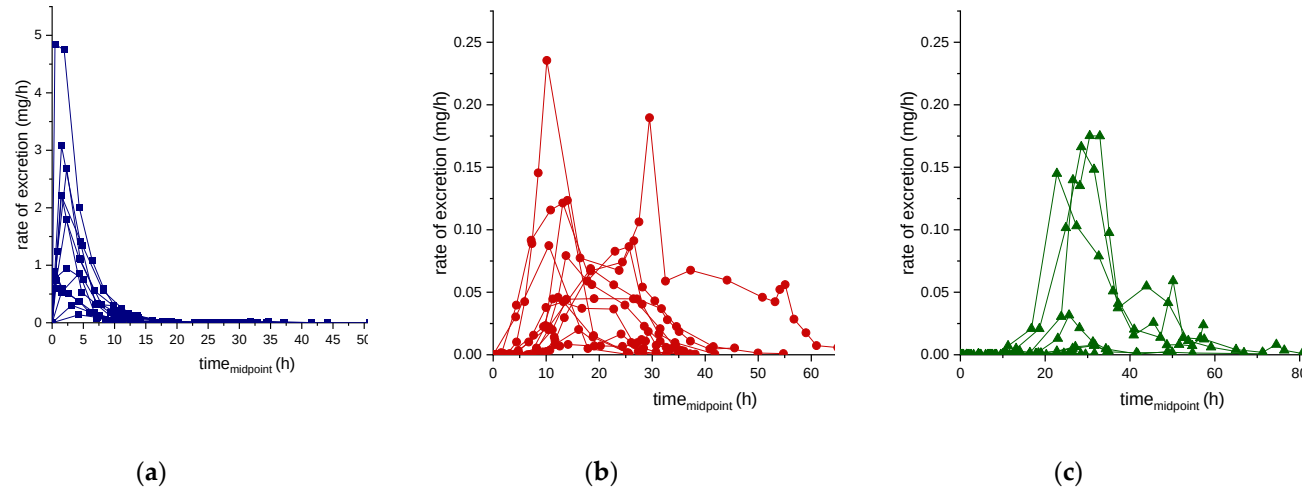


Figure 3. Urinary excretion profile (rate of excretion-midpoint time curve) of ecdysterone (a), 14-deoxy ecdysterone (b) and 14-deoxy poststerone (c) following a single dose administration of 50 mg of pure ecdysterone in humans (n=12).

Following a single dose administration of 50 mg of ecdysterone, the parent compound resulted to be the most abundant analyte in all post administration urines. Its maximum concentration was detected in the 2.8-8.5 h urines ranging from 4.4-30.0 µg/mL. The maximum excretion rate (mg/h) was detected in the 0.4-4.4 h urines ranging from 0.1-4.8 mg/h. Ecdysterone was detectable in the urines after 45 minutes from the administration and for more than 2 days (58h).

The metabolite 14d-ecdysterone was detectable as well in all urines analyzed. The maximum concentration was detected in the 8.5-39.5 h urines ranging from 0.1-6.0 µg/mL. The maximum excretion rate was determined in the 10.1-29.5 h urines ranging from 0.02-0.24 mg/h. 14d-ecdysterone was already detected after 2.95 h and remained detectable for about 3 days (75h).

In contrast to the parent compound and to the 14d-ecdysterone, 14d-poststerone was only detected in 10 out of 12 subjects. For 5 volunteers it was possible to obtain an excretion profile curve.

The maximum concentration was detected in the 23.3-41.3 h urines ranging from 0.1-1.5 µg/mL. The detection window of 14d-poststerone ranged from 8.50 to 97 h.

The mean (n=12) of the maximum urinary concentration (C_{max}), time to maximum urinary concentration (T_{max}), maximum urinary rate of excretion (RE_{max}) and maximum

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2021 doi:10.20944/preprints202105.0106.v1

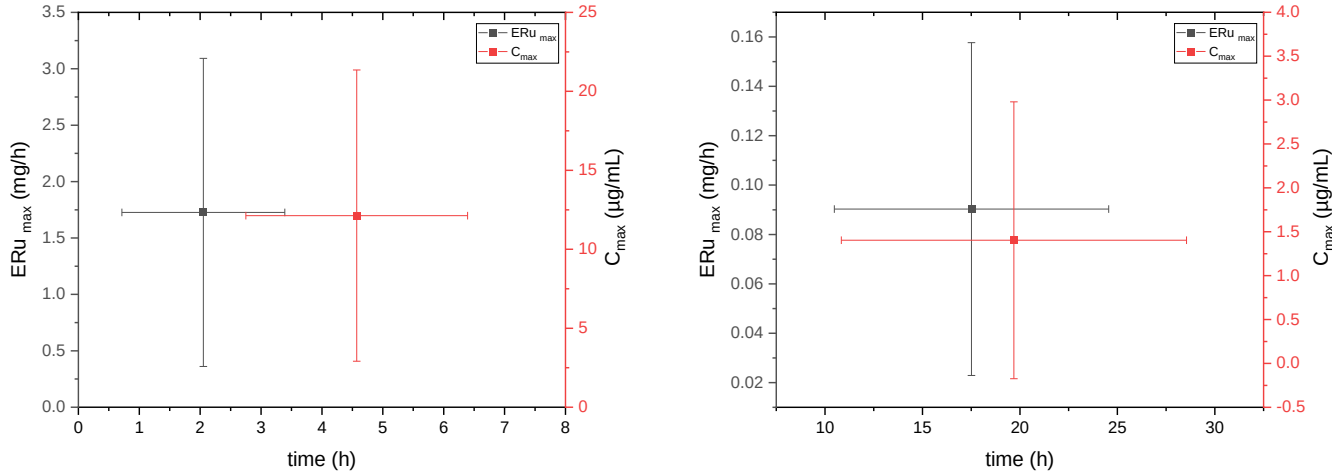
urinary middle point time (midpoint time$_{max}$) have been calculated and details are reported in Table 4.

Table 5. Urinary excretion profile parameters calculated as the mean of 12 subject ± standard deviation (SD) after administration of a single dose of 50 mg of pure ecdysterone.

Compound	Excretion profile parameters			
	C_{max} (µg/mL)	T_{max} (h)	RE_{max} (mg/h)	Midpoint time$_{max}$ (h)
Ecdysterone mean ± SD	12.1 ± 9.2	4.6 ± 1.8	1.7 ± 1.4	2.1 ± 1.3
14-deoxy-ecdysterone mean ± SD	1.4 ± 1.6	19.7 ± 8.9	0.1 ± 0.1	17.5 ± 7.0
14-deoxy-poststerone mean ± SD	0.8 ± 0.7*	30.1 ± 7.2*	0.1 ± 0.1*	27.8 ± 3.7*

*Calculated from n=5.

A graphic comparison of ecdysterone and 14d-ecdysterone excretion profile parameters using the rate of excretion values or the concentrations versus time is, reported in Figure 4.



(**a**) (**b**)

Figure 4. Comparison of results obtained using calculated concentration C_{max} or excretion rate ER_{max} versus time for ecdysterone (a) and 14d-ecdysterone (b). Each result is reported as the mean ± SD for all the subjects considered in the study (n=12).

2.3 Evaluation of urinary pharmacokinetic parameters: cumulative amount and half-life

For each subject considered in this study (n=12), the amount of ecdysterone, 14d-ecdysterone and 14d-poststerone excreted in urine (cumulative amounts, Du) after administration of 50 mg of pure ecdysterone have been calculated. The cumulative urinary excretion curves of ecdysterone, 14d-ecdysterone and 14d-poststerone, expressed as percentages relative to the dose administered (50mg) versus sampling time (hours) are displayed in Figure 5. The cumulative excretion percentages range from 2.8-47.2 for ecdysterone, from 0.4-6.1 for the 14d-ecdysterone and from 0.01-4.9 for 14d-poststerone. These results show that the cumulative excretion percentages of 14d-ecdysterone and 14d-poststerone are much lower than the one obtained for ecdysterone, which was excreted in urines faster than the metabolites (Figure 3 and 5).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2021 doi:10.20944/preprints202105.0106.v1

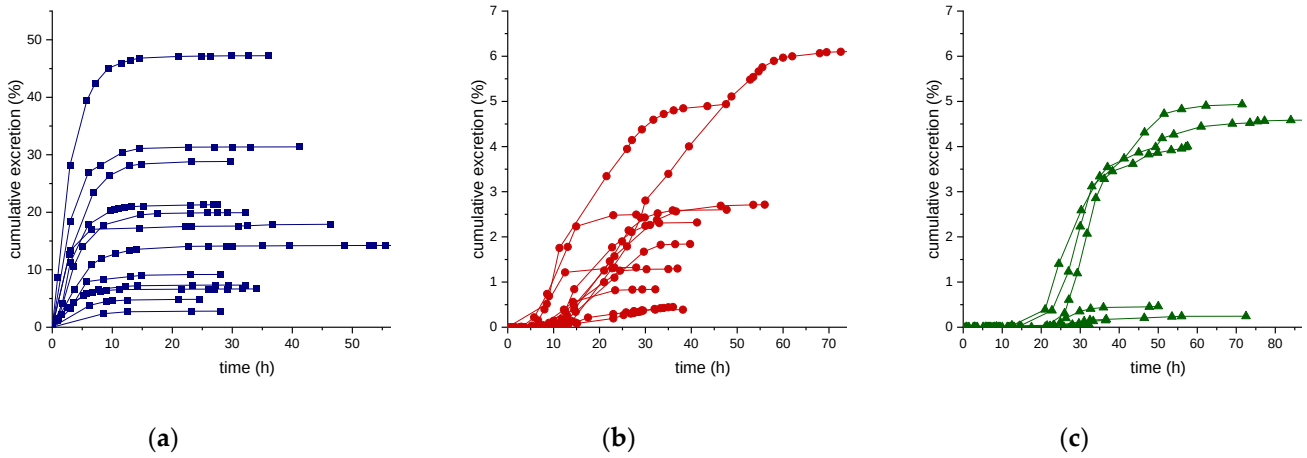


Figure 5. Cumulative urinary excretion curve of ecdysterone (a), 14d-ecdysterone (b) and 14d-poststerone (c) following a single dose administration of 50 mg pure ecdysterone in male and female (n=12).

The distributions of cumulative urinary excretion percentage of ecdysterone, 14d-ecdysterone and 14d-poststerone are reported as box-plots in Figure 6. The mean values correspond to 18 % with a SD of ±13, 2.3±1.74 and 1.5±2.1, respectively.

A comparison of the urinary cumulative percentages of ecdysterone, 14d-ecdysterone and 14d-poststerone after administration of 50 mg of pure ecdysterone in male and female has been performed using a T-test. No significant differences between male and female ($p \leq 0.05$) have been reported for ecdysterone and 14d-ecdysterone, while significant differences ($p \leq 0.05$) are reported for the metabolite 14d-poststerone. Results are reported in the boxplot in Figure 6.

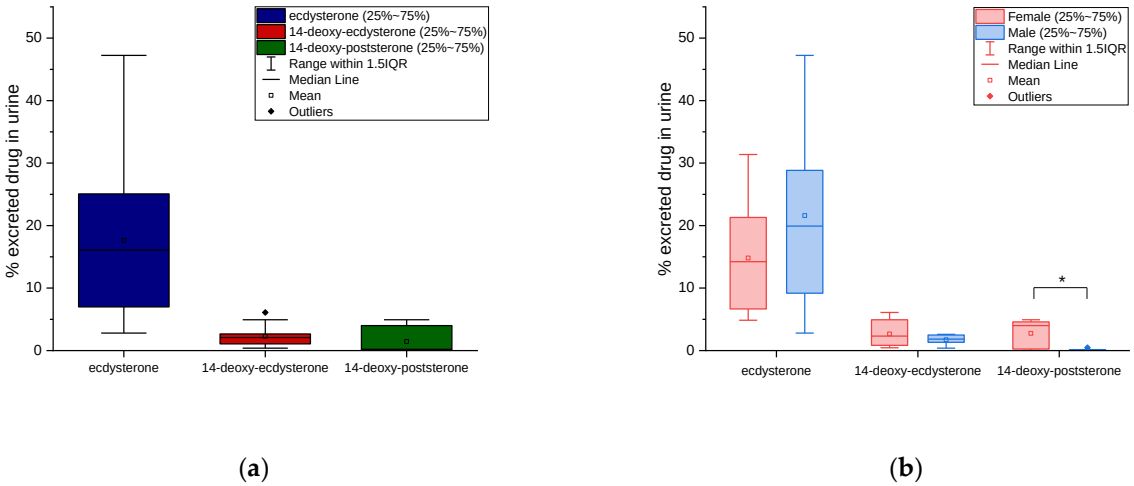


Figure 6. Box-plots of urinary cumulative excretion for ecdysterone, 14d-ecdysterone and 14d-poststerone (a) and their comparison in male and female after administration of 50 mg of ecdysterone (b); *significantly different $p \leq 0.05$ (b).

The half-life of ecdysterone, 14d-ecdysterone and 14d-poststerone in post administration urines were calculated using two different methods: rate of excretion method and sigma-minus method. The calculated half-life for ecdysterone and 14d-ecdysterone were found similar and corresponded to about 3 hours. The half-life of 14d-poststerone instead was much longer using the rate of excretion than the sigma minus method.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2021 doi:10.20944/preprints202105.0106.v1

Details of the cumulative amount of the analyte and the metabolites excreted in urines as well as the calculated half-life are reported in Table 5. the ln-transformed excretion rate used to calculate the elimination rate constant (k) of ecdysterone and the corresponding half-life is displayed in Figure 7.

Table 5. Urinary pharmacokinetic parameters calculated as the mean of 12 subject ± standard deviation (SD) after administration of a single dose of 50 mg of pure ecdysterone.

Compound	Cumulative Du (mg)	Half life (h)	
		Rate of excretion	Sigma-minus
Ecdysterone			
mean ± SD	8.8 ± 6.6	3.4 ± 1.0	3.0 ± 1.0
14-deoxy-ecdysterone			
mean ± SD	1.1 ± 0.9	3.1 ± 1.3	2.2 ± 0.9
14-deoxy-poststerone			
mean ± SD	0.7 ± 1.1**	9.7 ± 9.5*	4.4 ± 1.0*

*Calculated from n=5; ** calculated from n=10

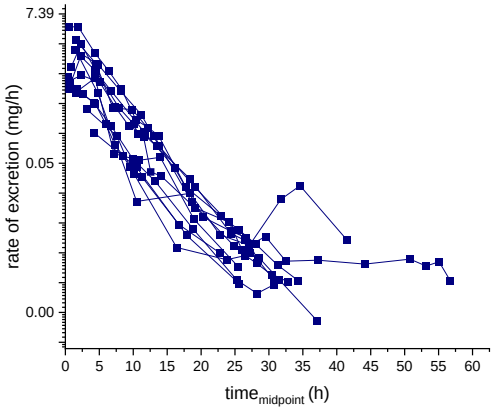


Figure 7. Ecdysterone ln-transformed excretion rate.

3. Discussion

In this study an LC-MS/MS method has been successfully developed and validated for the identification and quantitation of ecdysterone and its metabolites. After the administration of a single dose of 50 mg of pure ecdysterone to twelve healthy subjects (five males and seven females) the excretion profile and urinary pharmacokinetic parameters of ecdysterone and its metabolites were evaluated. Ecdysterone was the most abundant analyte detected in post administration urines. Its early detectability in urine (after 45 min post administration), indicates its rapid absorption and excretion. The kinetics of elimination of ecdysterone was evaluated using the log-linear rate of excretion versus the middle point time and corresponded to a first order.

Ecdysterone was detected in post-administration urines for more than 2 days and its C_{max} in urine was reached in the 2.8-8.5 h. From the results of the cumulative urinary excretion, we can assume that after 12 hours ecdysterone reaches a plateau indicating that it is almost completely eliminated, the maximum amount of ecdysterone in urines is reached and it corresponds to 18%. Ecdysterone has been found to have a short urinary half-life, which corresponds to 3.4 and 3.0 hours when using the rate of excretion and the sigma-minus method, respectively.

In this study the presence of the metabolite 14d-ecdysterone has been observed in the post administration urines and its formation has been confirmed for all the 12 subjects

 doi:10.20944/preprints202105.0106.v1

considered. The urinary detection of 14d-ecdysterone after administration of ecdysterone was previously reported by Brant and Parr et al. in humans [30, 31], Kumpun in mice [6] and Destrez in calves [39, 40]. Instead, Tsitsimpikou et al., reported the formation of a 2-deoxy ecdysterone and deoxy ecdysone as urinary metabolites [29]. Kumpun at al. reported the formation of a deoxy-metabolite as caused by gut bacteria [6]. This should be confirmed by further metabolic studies.

The 14d-ecdysterone was excreted later than the parent compound, probably due to its less polar physico-chemical characteristics; it was detected in post administration urines for about 3 days reaching the C_{max} in the 8.5-39.50 h urines.

Unlike ecdysterone, that after reaching the maximum excretion rate showed a decline to the base level, in several subjects, a first increase of the excretion rate of 14d-ecdysterone to a maximum level was followed by a second peak (Figure 3). Thus, it might be assumed that there is a rate-limiting step in pharmacokinetics process of 14d-ecdysterone or that it remains longer in other compartments before to be excreted in urine.

Results from the cumulative excretion of 14d-ecdysterone, in contrast to ecdysterone, show that the plateau is reached at different times in the different subjects considered. Specifically, in the 58.3% of the subjects the plateau is reached between 25-40 hours, in the 25% between 15 to 25 hours and in the remaining 16.6% between 40-62 hours. These results indicate an inter-individual variability in the formation, absorption and excretion rate of the metabolite. The maximum amount of 14d-ecdysterone reached in urines corresponds to 2.3 % (mean, n=12). As for ecdysterone, a short half-life was observed also for 14d-ecdysterne calculated as 3.1 and 2.2 hours using the rate of excretion and the sigma-minus method, respectively.

14d-Poststerone was detected as new metabolite in humans. Its identity was confirmed in post-administration human urines by comparison with authentic reference material. The 14d-poststerone was detectable in post-administration urines for about 4 days and the C_{max} was reached in the 23.3-41.3 h urines. The maximum amount excreted in urines corresponds to 1.5%. In mice, Kumpun et al. [6] already reported it as metabolite of ecdysterone as well. Unlike ecdysterone parent compound and 14d-ecdysterone, 14d-poststerone was identified in 10 out of 12 subjects only. Analogously to the formation of progestins form cholesterol in human steroid biosynthesis, 14d-poststerone may be generated by side chain cleavage. Postulated by Kumpun et al., this may be catalyzed either by a cytochrome P450 enzyme or generated by gut microorganisms [29].

As consequence, if these reactions are caused by the gut bacteria, individual variability in the metabolic profile needs to be considered. No correlation between the concentration of 14d-ecdysterone and 14d-poststerone was observed. To understand whether the latter can be selected as a target metabolite for an ecdysterone administration, further investigations are needed.

The cumulative excretion percentage of 14d-poststerone reached a plateau between 36 to 61 hours post-dosage. Similar to the trend of the excretion rate of 14d-ecdysterone, a small increase in excretion rate of 14d-poststerone, after the maximum peak was achieved, has been observed. The half-life of 14d-poststerone calculated using the excretion rate method corresponds to 9.7 ± 9.5 hours while with the sigma-minus it corresponds to 4.4 ± 1.0 hours. This can be explained as a result of the fluctuation observed during the elimination phase influencing the linearity of the line constructed to obtain the k-value, which is used to calculate the half-life.

The results of C_{max} obtained in this study show that there is a high inter-individual variability in the excretion of ecdysterone and its metabolites. Statistical evaluation was conducted to compare percentage of excreted ecdysterone, 14d-ecdysterone, and 14d-poststerone between female and male. No significant difference was found for ecdysterone and 14d-ecdysterone while for 14d-poststerone a significant difference ($p \leq 0.05$) between female and male was observed.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2021 doi:10.20944/preprints202105.0106.v1

Excretion profile results obtained when using the rate of excretion or the concentration, indicate that the knowledge of urinary information (e.g. volume, collection time) can influence the point in which the highest concentration or excretion rate will be detected.

In total, up to 50.3% (mean= 21.1%, n=12) of administered dose was recovered in urine (as a parent drug and metabolites). The remaining dose might be unabsorbed due to low bioavailability or excreted by other pathways such as biliary excretion, sweat, saliva, feces or it can also be transformed to another metabolite which was not yet detected and quantified within this study.

4. Materials and Methods

4.1. Chemicals and Reagents

Reference standards of ecdysterone (2β,3β,14α,20β,22R,25-hexahydroxy-5β-cholest-7-en-6-one, parent compound, **PC**) was purchased from Steraloids (Newport, RI, USA) while Ponasterone (2β,3β,14α,20β,22R-pentahydroxy-5β-cholest-7-en-6-one, used as internal standard, **ISTD**), was obtained from Santa Cruz Biotechnology, Inc. (Heidelberg, Deutschland). Alpha-14-deoxy ecdysterone (**M1**) and alpha-14-deoxy poststerone (**M2**) were purchased from Extrasynthese (Genay Cedex, France).

4.2. Oral administration of ecdysterone and urine collection

The study was approved by the ethical committee of the German Sport University Cologne and carried out following the regulations of the Helsinki declaration. Twelve healthy subjects (7 females and 5 males) with a mean age (standard deviation [SD]) of 26 (3.1) y, weight of 74 (12.0) kg and height 174 (8.3) cm participated in the study. A single-dose of 50 mg of pure ecdysterone has been administered to the subjects. All doses were administered at 9.00 a.m. in the morning. The urine samples were collected one day before (blank samples) and five days after the administration of ecdysterone. Sampling time (h) and urine volume (mL) were recorded. Aliquots of urine samples were stored frozen at -18° C until analysis.

4.3. Standard solutions and quality control samples

Stock solutions of ecdysterone, ponasterone (ISTD), 14d-ecdysterone and 14d-poststerone were prepared in methanol at a concentration of 1 mg/mL. Working solutions corresponding to 1000, 500, 250 µg/mL were prepared by diluting stock solution of ecdysterone, 14d-ecdysterone and 14d-poststerone in methanol and were used for preparation of the calibrants. Serial dilution with appropriate amount of working solutions were prepared in the pooled blank urines (matrix matched standards) to obtain the final concentrations of 1, 2.5, 5, 10, 25, 50, 100, 250, 500, 1000, 2500, 5000 ng/mL of ecdysterone, and concentrations ranging from 1 to 1000 ng/mL for 14d-ecdysterone and 14d-poststerone, to prepare the respective calibration standard ranges. Appropriate amount of a working solution of ponasterone (10 µg/mL) was prepared and used to spike the blank urines obtaining a final concentration of 100 ng/mL in all the calibrants.

Quality control (QC) sample were independently prepared at 4 different levels of concentration; for ecdysterone 1 ng/mL (LQC), at 250 ng/mL (MQC1), 2500 ng/mL (MQC2) and at 5000 ng/mL (HQC). Instead, for 14d-ecdysterone and 14d-poststerone the QC samples have been prepared at 1 ng/mL (LQC), 50 ng/mL (MQC1), 500 ng/mL (MQC2) and at 1000ng/mL (HQC).

4.4. Sample preparation

Urine samples (200 µL) spiked with 10 µL of the ISTD ponasterone (working solution 10 µg/mL) and diluted to 1 mL with methanol:water (10:90, v/v). The tubes were vortex-mixed and then centrifuged at 9677 RCF for 8 minutes.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2021 doi:10.20944/preprints202105.0106.v1

The supernatants were transferred to autosampler vials and analyzed.

4.5. Urine analysis

The analysis of ecdysterone and its metabolite in calibration and urine samples was performed by LC-MS/MS on an Agilent 1200 Infinity series coupled to an Ultivo triple quadrupole tandem MS system utilizing a Jet Stream electrospray ionization (ESI) source and Ion Funnel (Agilent Technologies GmbH, Waldronn,Germany).

4.5.1. Chromatographic conditions

Chromatographic separation was achieved with an Agilent Eclipse Plus C18 column (2.1 mm x 50 mm, particle size 1.8 µm). The gradient program starts at 12% of eluent B and linear increases to 40% in 4 min, then to 98% in 1.20 min, 0.30 min hold, followed by 0.20 min equilibration at 12% of eluent B. The linear gradient was applied at a flow rate of 0.45 mL/min resulting in a total run time of 5.7 min plus 1 min for column equilibration. Solvent A comprised of aqueous formic acid (H2O:FoOH, 99.9:0.1, v/v), and acetonitrile:formic acid (ACN:FoOH, 99.9:0.1, v/v) was used as solvent B. The sample injection volume was 5 µL.

4.5.2. Mass spectrometric parameters

The mass spectrometer was operated in multiple reaction monitoring (MRM) acquisition mode using positive ionization (ESI+). The protonated molecular ion [M+H]+ for ecdysterone, ponasterone, 14d-ecdysterone and 14d-poststerone were detected at m/z 481.3 for ecdysterone, at 465.3 m/z for ponasterone and 14d-ecdysterone (isomers) and at 347.2 m/z for 14d-poststerone.

Source and MRM optimization were performed using MassHunter software (Agilent Technologies Inc. Santa Clara, CA, USA). As resulting parameters capillary voltage of 4000 V, nozzle voltage of 500 V, drying gas flow of 5 L/min (nitrogen) at 150°C, sheath gas flow of 12 L/min (nitrogen) at 375°C and nebulizer pressure of 30 psi (nitrogen) were used for the experiments. In Table 6 are reported the mass spectrometric parameters for the MRM transition of ecdysterone, ponasterone, alpha-14-deoxy-20-hydroxy ecdysone and alpha-14-deoxy poststerone. The chromatograms and mass spectra are reported as supplemental material.

Table 6. Mass spectrometric parameters for MRM transitions for ecdysterone, its metabolites and the ISTD.

Analytes	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Collision energy	Polarity
Ecdysterone					
quantifier	2.991	481.3	445.3	13.0	positive
qualifier		481.3	427.3	13.0	positive
qualifier		481.3	371.2	9.0	positive
qualifier		481.3	80.9	57.0	positive
14-deoxy ecdysterone					
quantifier	3.519	465.3	303.2	21.0	positive
qualifier		465.3	80.9	53.0	positive
qualifier		465.3	285.2	25.0	positive
qualifier		465.3	267.2	29.0	positive
qualifier		465.3	104.9	73.0	positive
qualifier		465.3	90.9	89.0	positive
14-deoxy poststerone					
quantifier	4.068	347.2	329.1	16.0	positive
qualifier		347.2	173.0	28.0	positive
qualifier		347.2	90.9	68.0	positive
qualifier		347.2	105.0	56.0	positive
Ponasterone					

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2021 doi:10.20944/preprints202105.0106.v1

quantifier	4.805	465.3	447.3	9.0	positive
qualifier		465.3	90.9	89.0	positive
qualifier		465.3	80.9	37.0	positive

4.6 Evaluation of excretion profile and pharmacokinetic parameters in urines

In this study, the excretion profile, the cumulative amount excreted in urines and half-life of ecdysterone and its metabolites, after a single-dose administration of 50 mg of pure ecdysterone were evaluated according to [41].

To obtain the excretion rates the equation 1 was applied:

$$E\text{rate,i} = \frac{Ci\ x\ Vi}{(ti - ti{-}1)} \quad (1)$$

For each urine sample, the calculated concentration (Ci, ng/mL) of ecdysterone, 14d-ecdysterone, and 14d-poststerone was adjusted as a function of the volume of urine collected (Vi, mL), obtaining the correspondent amount of drug in urine (expressed in ng), which was divided by the interval between the time values of the sampling and the previous sampling (ti-ti-1).

The excretion profile curves of ecdysterone and its metabolites for each subject were then obtained plotting the calculated rate of excretion values (ng/h) versus time (middle point of sample collection, hours) or simply the calculated concentrations (ng/mL) versus time (sampling time, hours). The first collected interval starts at 0 hours after the oral administration of ecdysterone at 9 a.m.

For each sample the amount of drug excreted in urine was calculated and added to the amount of drug recovered in the previous urine sample, to obtain the cumulative urinary drug excretion, which was plotted in a graph versus the sampling time (hours).

The half-life using the elimination rate constant (k) was calculated applying the equation 2:

$$t1/2 = \frac{ln\ (2)}{k} \quad (2)$$

Two different methods were used to calculate the elimination rate constant (k); in the rate of excretion method the k was obtained from the slope of the elimination phase of the ln-transformed excretion curve (Figure 7).

In the sigma minus method, k was obtained from the slope of the elimination phase of the ln-transformed remaining drug to be excreted.

Mass Hunter Quant Software from Agilent was used for data acquisition and analysis. Origin Pro 9.1 software (OriginLab Corporation, Northampton, MA, USA) was used for data visualization and statistical treatment of the data.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

Author Contributions: Conceptualization, M.P. and F.B.; methodology, G.A., T.Y., B.W., E.I.; software, B.W.; validation, G.A., T.Y.; formal analysis, G.A., T.Y.; investigation, G.A., T.Y.; resources, E.I., P.D., M.P.; data curation, G.A.; writing—original draft preparation, G.A.; writing—review and editing, M.P., F.B., M.M.; visualization, G.A., T.Y.; supervision, M.P., F.B., M.M.; project administration, M.P.; funding acquisition, M.P., F.B., M.M., B.W., P.D., and X.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the World Anti-Doping Agency (WADA, grant number 18C18MP. The APC was funded by the publication fund of Freie Universitaet Berlin".

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of German Sport University Cologne (protocol code 148/2018).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Raw data are stored at the authors.

Acknowledgments: We acknowledge financial support by the World Anti-Doping Agency and the Open Access Publication Fund of the Freie Universität Berlin.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Otaka, T., S. Okui, and M. Uchiyama, *Stimulation of protein synthesis in mouse liver by ecdysterone.* Chemical & pharmaceutical bulletin, 1969. **17**(1): p. 75.
2. Slama, K. and R. Lafont, *Insect hormones-ecdysteroids: Their presence and actions in vertebrates.* European Journal of Entomology, 1995. **92**(1): p. 355-377.
3. Dinan, L., *The Karlson Lecture. Phytoecdysteroids: What use are they?* Archives of Insect Biochemistry and Physiology, 2009. **72**(3): p. 126-141.
4. Parr, M.K., et al., *Estrogen receptor beta is involved in skeletal muscle hypertrophy induced by the phytoecdysteroid ecdysterone.* Molecular Nutrition & Food Research, 2014. **58**(9): p. 1861-1872.
5. Tchoukouegno, N.S., *Bioactivity of plants secondary metabolites: Estrogenic, cytotoxic and anabolic effects on estrogen target organs of an extract of Erythrina excelsa and Ecdysterone.* PhD, German Sport University, Cologne, 2013.
6. Kumpun, S., et al., *The metabolism of 20-hydroxyecdysone in mice: relevance to pharmacological effects and gene switch applications of ecdysteroids.* J Steroid Biochem Mol Biol, 2011. **126**(1-2): p. 1-9.
7. Wilborn, C.D., et al., *Effects of methoxyisoflavone, ecdysterone, and sulfo-polysaccharide supplementation on training adaptations in resistance-trained males.* Journal of the International Society of Sports Nutrition, 2006. **3**(2): p. 19-27.
8. M McBride, J., *Phytoecdysteroids: A Novel, Non-Androgenic Alternative for Muscle Health and Performance.* Journal of Steroids & Hormonal Science, 2013. **s12**(01).
9. Gorelick-Feldman, J., et al., *Phytoecdysteroids increase protein synthesis in skeletal muscle cells.* Journal of agricultural and food chemistry, 2008. **56**(10): p. 3532.
10. Dinan, L., *Phytoecdysteroids: biological aspects*. 2001. p. 325-339.
11. Dinan, L. and R. Lafont, *Effects and applications of arthropod steroid hormones (ecdysteroids) in mammals.* Journal of Endocrinology, 2006. **191**(1): p. 1-8.
12. Lafont, R. and L. Dinan, *Practical uses for ecdysteroids in mammals including humans: an update.* Journal of Insect Science, 2003. **3**.
13. Courtheyn, D., et al., *Recent developments in the use and abuse of growth promoters.* Analytica Chimica Acta, 2002. **473**(1-2): p. 71-82.
14. Tóth, N., et al., *20-Hydroxyecdysone increases fiber size in a muscle-specific fashion in rat.* Phytomedicine, 2008. **15**(9): p. 691-698.
15. Bathori, M., et al., *Phytoecdysteroids and anabolic-androgenic steroids--structure and effects on humans.* Curr Med Chem, 2008. **15**(1): p. 75-91.
16. Sláma, K., et al., *Insect hormones in vertebrates: Anabolic effects of 20-hydroxyecdysone in Japanese quail.* Experientia, 1996. **52**(7): p. 702-706.
17. Okui, S., et al., *Stimulation of Protein Synthesis in Mouse Liver by Insect-Moulting Steroids.* Chemical and Pharmaceutical Bulletin, 1968. **16**(2): p. 384-387.
18. Arking, R. and E. Shaaya, *Effect of ecdysone on protein synthesis in the larval fat body of Calliphora.* Journal of Insect Physiology, 1969. **15**(2): p. 287-296.
19. Burdette W, C.R., *Effect of ecdysone on the incorporation of 14C-Leucine into hepatic protein in vitro.* Proc Soc Exp Biol Med, 1963. **12**: p. 216–217.
20. Parr MK, H.O., Ngueu ST, Fritzemeier K-H, Muhn P, Diel PR *Estrogen receptor beta mediated anabolic effects -insights from mechanistic studies on the phytoecdysteroid ecdysterone and selective ligands.* Endocr Rev, 2013. **v 10:SAT-340**.

21. Parr MK, W.G., Naß A, Ambrosio G, Botrè F, Diel PR, *ER-beta mediated action of dietary supplement ingredient edcysterone confirmed by docking experiments.* Endocrine Reviews, 2015. **FRI-270**.
22. Parr MK, M.-S.A., *Pharmacology of doping agents—mechanisms promoting muscle hypertrophy.* AIMS Molecular Science, 2018. **5**(2): p. 145-155.
23. Parr, M.K., et al., *Ecdysteroids: A novel class of anabolic agents?* Biol Sport, 2015. **32**(2): p. 169-73.
24. Chermnykh, N.S., et al., *The action of methandrostenolone and ecdysterone on the physical endurance of animals and on protein metabolism in the skeletal muscles.* Farmakol Toksikol, 1988. **51**(6): p. 57-60.
25. World Anti-doping Agency, *The 2020 Monitoring Program.* Available online at https://www.wada-ama.org/sites/default/files/wada_2020_english_monitoring_program_.pdf (last accessed December 1st 2019).
26. Isenmann, E., et al., *Ecdysteroids as non-conventional anabolic agent: performance enhancement by ecdysterone supplementation in humans.* Arch Toxicol, 2019. **93**(7): p. 1807-1816.
27. Lafont, R., et al., *Ecdysteroid metabolism: A comparative study.* Insect Biochemistry, 1986. **16**(1): p. 11-16.
28. Ramazanov, N., Z. Saatov, and B. Syrov, *Study of ecdysterone metabolites isolated from rat urine.* Chemistry of Natural Compounds, 1996. **32**(4): p. 545-549.
29. Tsitsimpikou, C., et al., *Study of excretion of ecdysterone in human urine.* Rapid Communications in Mass Spectrometry, 2001. **15**(19): p. 1796-1801.
30. Brandt, F., *Pharmakokinetik und Metabolismus des 20-Hyroxyecdysons im Menschen.* PhD, Philipps-Universität Marburg, Marburg, 2003.
31. Parr, M.K., et al., *Targeting the administration of ecdysterone in doping control samples.* Forensic toxicology, 2020. **38**(1): p. 172-184.
32. European Medicines Agency, *Guideline on Bioanalytical Method validation.* 2011.
33. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, *Draft version. M10: Bioanalytical Method Validation*. 2019.
34. International Conference on Harmonization of Technical Requirements fo Registration of Pharmaceuticals for Human Use, *Validation of Analytical Procedurs: Text and Methodology Q2(R1).* 2005.
35. Keizer, R.J., et al., *Incorporation of concentration data below the limit of quantification in population pharmacokinetic analyses.* Pharmacol Res Perspect, 2015. **3**(2): p. e00131.
36. Hecht, M., et al., *Utilization of data below the analytical limit of quantitation in pharmacokinetic analysis and modeling: promoting interdisciplinary debate.* Bioanalysis, 2018. **10**(15): p. 1229-1248.
37. Dorababunn, M., *Pharmacokinetic Modeling of Data with Below Quantification Limit.* Journal of bioequivalence & bioavailability, 2012. **4**(2).
38. Senn, S., N. Holford, and H. Hockey, *The ghosts of departed quantities: approaches to dealing with observations below the limit of quantitation.* Statistics in medicine, 2012. **31**(30): p. 4280-4295.
39. Destrez, B., et al., *Detection of 20-hydroxyecdysone in calf urine by comparative liquid chromatography/high-resolution mass spectrometry and liquid chromatography/tandem mass spectrometry measurements: application to the control of the potential misuse of ecdysteroids in cattle.* Rapid communications in mass spectrometry, 2008. **22**(24): p. 4073-4080.
40. Destrez, B., et al., *Detection and identification of 20-hydroxyecdysone metabolites in calf urine by liquid chromatography-high resolution or tandem mass spectrometry measurements and establishment of their kinetics of elimination after 20-hydroxyecdysone administration.* Anal Chim Acta, 2009. **637**(1-2): p. 178-84.
41. Shargel L, W.-P.S.a.Y.A., *Applied Biopharmaceutics and Pharmacokinetics.* 5th ed.: McGraw-Hill Medical, 2004.