Short communication

Drug Testing and Analysis

Received: 19 May 2011 Revised: 19 July 2011 Accepted: 2 August 2011 Published online in Wiley Online Library: 17 November 2011

(wileyonlinelibrary.com) DOI 10.1002/dta.352

Zeranol: doping offence or mycotoxin? A case-related study

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Zeranol ((7R,11S)-7,15,17-trihydroxy-11-methyl-12-oxabicyclo[12.4.0]octadeca-1(14),15,17-trien-13-one, also referred to as 7α-zearalanol, Ralone®, Frideron®, Ralgro®, etc.) is a semi-synthetic estrogenic veterinary drug with growth-promoting properties. Its use regarding animal husbandry has been prohibited in the European Union since 1981 and, due to its anabolic effects, it is further recognized as a banned substance in sport. Numerous studies were conducted concerning the identification of the illicit application of zeranol to domestic livestock. These studies also considered the natural occurrence of zeranol as a metabolite of the mycotoxin zearalenone and the issue of differentiating both scenarios, i.e. illegal use or unintended contamination. Human sports drug testing authorities are facing comparable challenges since the deliberate misuse of the (for human application non-approved) drug should be discriminated from adverse analytical findings resulting from the biotransformation of the mycotoxin zearalenone possibly ingested with contaminated food. The active drug (zeranol), its major human metabolites (zearalanone, 7β-zearalanol) and the mycotoxin (zearalenone) plus its major and unique metabolic products (α-zearalenol, β-zearalenol) have been monitored in routine doping controls by means of validated gas chromatography-(tandem) mass spectrometry (GC-(MS/)MS) methods since 1996, and between 2005 and 2010 four samples providing suspicious signals were detected. In agreement with literature data, *in vitro* metabolism studies demonstrated the metabolic pathway from zearalenone towards zeranol (and common metabolites). In contrast, an administration study urine sample (collected after oral application of 20 mg of zeranol) yielded only ultra-trace amounts of zearalenone and its characteristic metabolites, which supported the assumption that a mycotoxin contamination caused the finding of zeranol in the doping control specimens rather than a misuse of the anabolic agent. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: sport; doping; mass spectrometry; anabolics; metabolite pattern

Introduction

The non-steroidal growth promoter zeranol ((7R,11S)-7,15,17-trihydroxy-11-methyl-12-oxabicyclo[12.4.0]octadeca-1(14),15,17-trien-13-one, Figure 1, 1), which is also referred to as 7α-zearalanol, Ralone®, Frideron®, Ralgro®, etc.), is an approved drug for stock farming in the USA and Canada but prohibited for the same purpose within the European Union.[1,2] Consequently, various studies have been conducted over the last three decades to efficiently and sensitively screen for trace amounts of zeranol in Europe. The analyses were conducted using gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-(tandem) mass spectrometry (LC-(MS/)MS) employing different animal matrices including urine, muscle, and liver tissue.[3–6] In this context, the natural occurrence of zeranol was observed and studied,[7] outlining the production of the structurally closely related mycotoxins zearalenone, α- and β-zearalenol (Figure 1, **4**, **5**, and **6**, respectively) by selected *Fusarium* spp. fungi species such as *F. graminearum*, *F. culmorum*, *F. crookwellense*, etc.,[8] which have been the source of zeranol precursors for industrial pharmaceutical preparation. These fungi colonize maize, wheat, oats, and barley, for example, and the consumption of contaminated pasture was shown to result in analytical findings of zeranol and its major metabolites in different animal species.[2,9,10]

Due to its growth promoting and assumed anabolic effects in humans, zeranol (**1**) also belongs to the list of substances prohibited in sports as classified by the World Anti-Doping Agency (WADA).[11] Since more than 15 years, zeranol has been tested in routine doping controls,[12,13] and comparable to the issue of animal husbandry described, sports drug testing might yield adverse analytical findings for zeranol resulting from natural contamination as several reviews have summarized the worldwide occurrence of zearalenone in agricultural commodities.[8,14] Hence, analytical tools are required to enable the differentiation of deliberate misuse of zeranol as a growth-promoting agent from unintended ingestion due to food contamination. An approach established for food quality assurance considers the metabolite pattern of zeranol and its natural precursor zearalenone,[7,15] which could also be particularly useful for doping control purposes since the biotransformation of zeranol in humans as studied *in vitro*[16,17] as well as *in vivo*[18] was found to be significantly different from that of zearalenone.

In the present report, the finding of four zeranol-containing doping control samples is presented and discussed in the context of illicit use and potential food contamination. Detected by GC-(MS/)MS, the metabolic profile was determined and the potential of *in vitro* inter-conversion of zeranol, zearalenone, and corresponding phase I metabolites was evaluated using human liver microsomal preparations.

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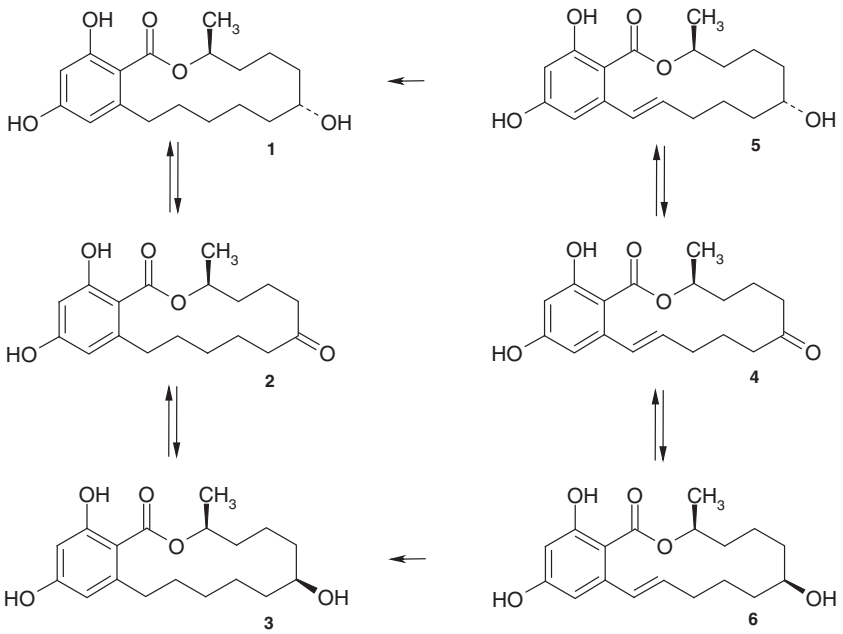

Figure 1. Chemical structures and metabolic correlations of zeranol (α-zearalanol, **1**), zearalanone (**2**), taleranol (β-zearalanol, **3**), zearalenone (**4**), α-zearalenol, (**5**), and β-zearalenol (**6**).

Experimental

Chemicals, reagents, and reference substances

N-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Chemische Fabrik Karl Bucher (Waldstetten, Germany), NADPH and β-glucuronidase from *E. coli* were obtained from Roche Diagnostics (Mannheim, Germany), and human liver microsomal fraction were from BD Gentest (Woburn, MA, USA). MiliQ water was used for all aqueous buffers and all solvents were of analytical grade and supplied by Merck (Darmstadt, Germany). The reference compounds zeranol, taleranol, zearalanone, zearalenone, α- and β-zearalenol were purchased from the National Measurement Institute (NMI, Sydney, Australia).

Urine specimens

Urine samples included more than 80 000 routine doping control specimens from elite athletes (analyzed between 2004 and 2010 in the Cologne doping control laboratory). The four suspicious samples observed in this period of time originated from in-competition tests and concerned three female and one male athlete. An administration study urine specimen (WADA external quality assurance scheme samples (EQAS) collected after oral administration of 20 mg of zeranol) was employed for comparison purposes.

Urine sample preparation

In order to recover phase I metabolites from the urine samples, 3 ml aliquots were prepared according to established doping control procedures.[19] In brief, the screening protocol including an enzymatic hydrolysis step with β-glucuronidase followed by liquid-liquid extraction (LLE) and evaporation was used. Subsequently, subjection to trimethlylsilylation[20,21] followed for GC-(MS/)MS-based analysis.

In vitro metabolic assay and sample work-up

In order to assess potential inter-conversions of zeranol, zearalenone, and their major metabolites, *in vitro* metabolism studies were conducted with each of these compounds using a formerly reported *in vitro* assay based on human liver microsomal enzyme fractions as source of metabolizing enzymes.[22] Stock solutions of 100 μM substrate concentrations were prepared in methanol and stored at 4 °C. A volume of 50 μl of each analyte's solution was transferred into a test tube, evaporated in a vacuum centrifuge and reconstituted in 90 μl of 50 mM phosphate buffer (pH 7.4), containing 5 mM $MgCl_2$ and 5 mM NADPH. For the initiation of metabolism, 5 μl of microsomal liver enzymes (200 μg total protein content per sample), was added and the reaction was carried out for 2 h at 37 °C. For the discrimination of metabolic from non-metabolic transformations, different blank samples were prepared with each batch of incubation, i.e. a substrate blank (containing all reactants but not the substrate), a co-factor blank (containing all reactants but not NADPH) and an enzyme blank. Phase II metabolic reactions were not performed.

The incubations were terminated by adding 200 μl of ice-cold acetone and the precipitated protein removed by centrifugation of the samples at 17 000 x *g* and 4 °C for 5 min. The supernatant was transferred to a fresh test tube and acetone removed in a vacuum centrifuge. The resulting solution was further purified by solid phase extraction using OASIS HLB SPE extraction cartridges (Waters, Milford, MA), methanol elution (2 ml), concentration *in vacuo* and reconstitution of the residue in 100 μl of acetic acid 2%/acetonitrile (9:1, *v/v*), before analysis by LC-MS/MS.

Gas chromatography – (tandem) mass spectrometry

GC-(MS/)MS analyses were conducted using a Thermo (Dreieich, Germany) Trace GC Ultra connected to a TSQ Quantum XLS GC triple quadrupole MS. The GC was equipped with a J&W Scientific Ultra 1 column (inner diameter 0.2 mm, film thickness 0.11 μm, length 17 m) from Agilent Technologies (Waldbronn, Germany). A temperature program was used starting at 185 °C increasing by 4 °C/min to 234 °C and then by 40 °C to 310 °C. Helium was used as carrier gas (constant pressure at 17.2 psi), the injector and transfer line temperatures were set to 300 °C, and the ion

source temperature was 250 °C. A volume of 1.5 µl of the derivatized sample was injected in split mode (1:15), and compounds were detected after electron ionization (EI) at 70 eV. For MS/MS experiments, precursor ions were selected at a window width of 1 *m/z* unit, collision energies of 15–25 eV were applied, collision gas was argon (purity grade 5.0) and ion transitions were recorded in selected reaction monitoring (SRM) mode (Table 1).

Liquid chromatography – (tandem) mass spectrometry

In vitro metabolism samples were analyzed using a Thermo Accela liquid chromatrogaph interfaced to a Thermo Exactive mass spectrometer (Bremen, Germany). The MS was operated in negative ionization mode and calibrated using the manufacturer's calibration mixture (yielding a total of 7 reference masses). Mass accuracies < 5 ppm were accomplished for the period of analysis. The ionization voltage was −3.0 kV, the capillary temperature was set to 290 °C, and two MS settings were used throughout the analytical runs: (1) full-scan MS from *m/z* 100–600 at a resolution of 100 000 (FWHM), and (2) full-scan MS (*m/z* 50–600, resolution set to 25 000) with higher energy collision-induced dissociation (HCD) set to 20 V. Collision and damping gas was nitrogen delivered by a nitrogen generator (CMC Instruments, Eschborn, Germany). The LC was equipped with a Macherey-Nagel Pyramid RP C_{18} analytical column (2.0 x 50 mm, 3 µm particle size, Düren, Germany). Five mM ammonium acetate buffer (pH = 3.5) and acetonitrile were used as mobile phases A and B, respectively. The gradient started at 100% A with a flow rate of 250 µl/min and was linearly increased to 100% B in 11 min.

Table 1. Characteristic fragment ions of per-TMS-derivatives of compounds **1–6** resulting from GC-MS and -MS/MS experiments using electron ionization. Precursor-product ion pairs used for screening and confirmation purposes are written in bold.

	EI-MS	EI-MS/MS		
Cmp.	Fragment ions, *m/z* (rel. abundance)	Precursor (*m/z*)	CE[b]	Product ions, *m/z* (rel. abundance)
1 / 3	448 (7)[a]	433	20	433 (20)
	433 (65)			415 (18)
	415 (12)			**389** (21)
	389 (11)			337 (18)
	379 (13)			323 (24)
	335 (46)			**309** (58)
	307 (100)			**295** (100)
	295 (38)			279 (20)
	268 (27)			
2	**446** (61)[a]	446	10	446 (72)
	431 (29)			431 (30)
	361 (21)			371 (52)
	335 (25)			**361** (100)
	307 (48)			359 (24)
	225 (33)			321 (30)
	197 (37)			**317** (12)
	169 (100)			**306** (88)
4	444 (2)[a]	305	20	305 (83)
	429 (9)			**289** (100)
	305 (100)			**273** (70)
	289 (10)			259 (29)
	277 (15)			233 (18)
	197 (42)			215 (10)
	187 (74)			185 (12)
	155 (63)			141 (20)
5 / 6	**446** (68)[a]	446	10	446 (100)
	431 (51)			431 (28)
	413 (18)			375 (22)
	333 (46)			371 (32)
	307 (77)			**361** (68)
	305 (100)			**333** (86)
	260 (40)			**317** (62)
	225 (53)			**306** (54)
	197 (84)			
	183 (71)			
	169 (65)			
	147 (45)			

[a]molecular ion
[b]CE = collision energy (eV)

Results and discussion

Routine doping controls for the presence of zeranol and its metabolites were conducted by means of GC-MS/MS targeting the active drug, the oxidation product zearalanone, and the 7β-isomer of zeranol referred to as taleranol, (Figure 1, 2, and **3**, respectively) with estimated detection limits of 0.1–0.5 ng/ml. Due to the reported issue of potential zearalenone contamination of food (particularly cereal-derived products), the mycotoxins zearalenone (**4**), α-zearalenol (**5**), and β-zearalenol (**6**) were included in confirmatory analyses for zeranol-suspicious sports drug testing samples using diagnostic ion transitions (Figures 2a and 2b; Table 1). Over a period of six years, no adverse analytical finding was reported worldwide for zeranol in a doping control context; however, four specimens (three female athletes and one male athlete) provided doping control urine samples that contained zeranol (**1**), zearalanone (**2**), and taleranol (**3**). Upon re-analysis, also the mycotoxins zearalenone (**4**), α-zearalenol (**5**), and β-zearalenol (**6**) were detected as illustrated for one of these specimens in Figure 2c. In all cases, the isomers zeranol (**1**) and taleranol (**3**) were observed at 16.72 and 16.88 min, respectively, accompanied by the corresponding oxidation product zearalanone (**2**) at 16.82 min, which (as such) represent an adverse analytical finding according to WADA rules. Additionally, zearalenone (**4**, 16.87 min), α- and β-zearalenol (**5**, 17.21 and **6**, 17.35 min, respectively) occurred in all suspicious samples, requiring further considerations as to the origin of the prohibited substance in the athletes' urine. Estimating the quantity of the target compounds yielded the results presented in Table 2, which contradict the relative abundance of zeranol metabolites as found in a urine specimen measured after oral administration of the pharmaceutical product (Figure 2d; Table 2 E). The metabolism of zeranol after oral application primarily produces zearalanone (**2**) (approximately 19.5 ng/ml) and taleranol (**3**, approximately 17.8 ng/ml) while signals indicating the mycotoxin-typical compounds (Figure 1, **4–6**) were observed at the detection limit (ca. 0.2 ng/ml) or not detected at all (Table 2). In case of the suspicious doping control samples, the metabolic pattern was considerably different and in accordance with literature data describing the *in vivo* metabolism of zearalenone for several different species.[5,8,10,14] Here, most abundant analytes were zearalenone (**4**) and α-zearalenol (**5**), all between 4 and 30 ng/ml, while corresponding values for zeranol (**1**), taleranol (**3**), and zearalanone (**2**) were always below 2.5 ng/mL (Table 2). An informative way to outline major

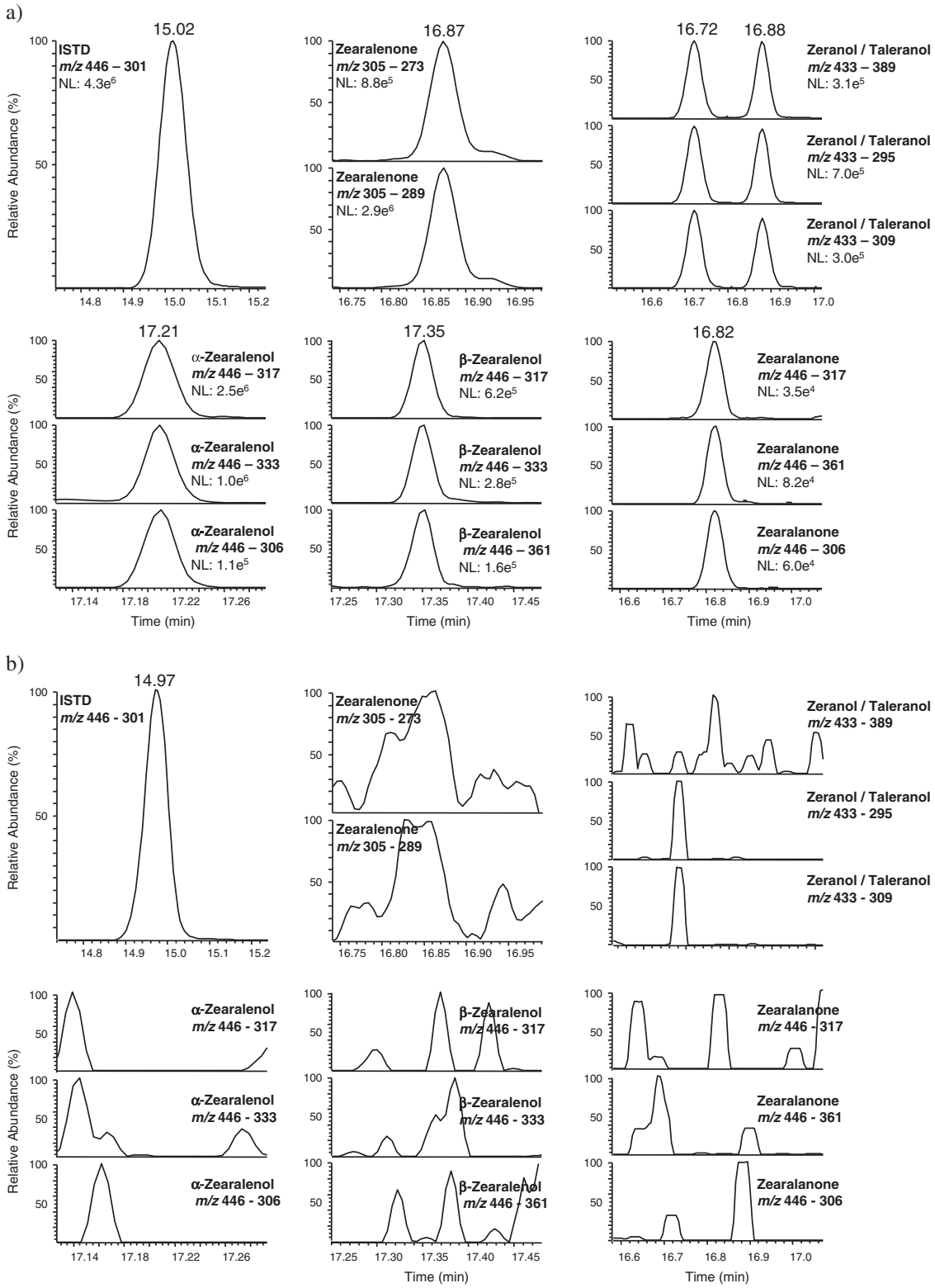

Figure 2. Extracted ion chromatograms of analyses showing (a) a urine sample spiked with the target analytes **1** and **3** at 5 ng/ml and all other (**2**, **4**–**6**) at 10 ng/ml; (b) a negative control urine sample (blank); (c) a doping control urine sample; and (d) a urine specimen collected after oral administration of zeranol.

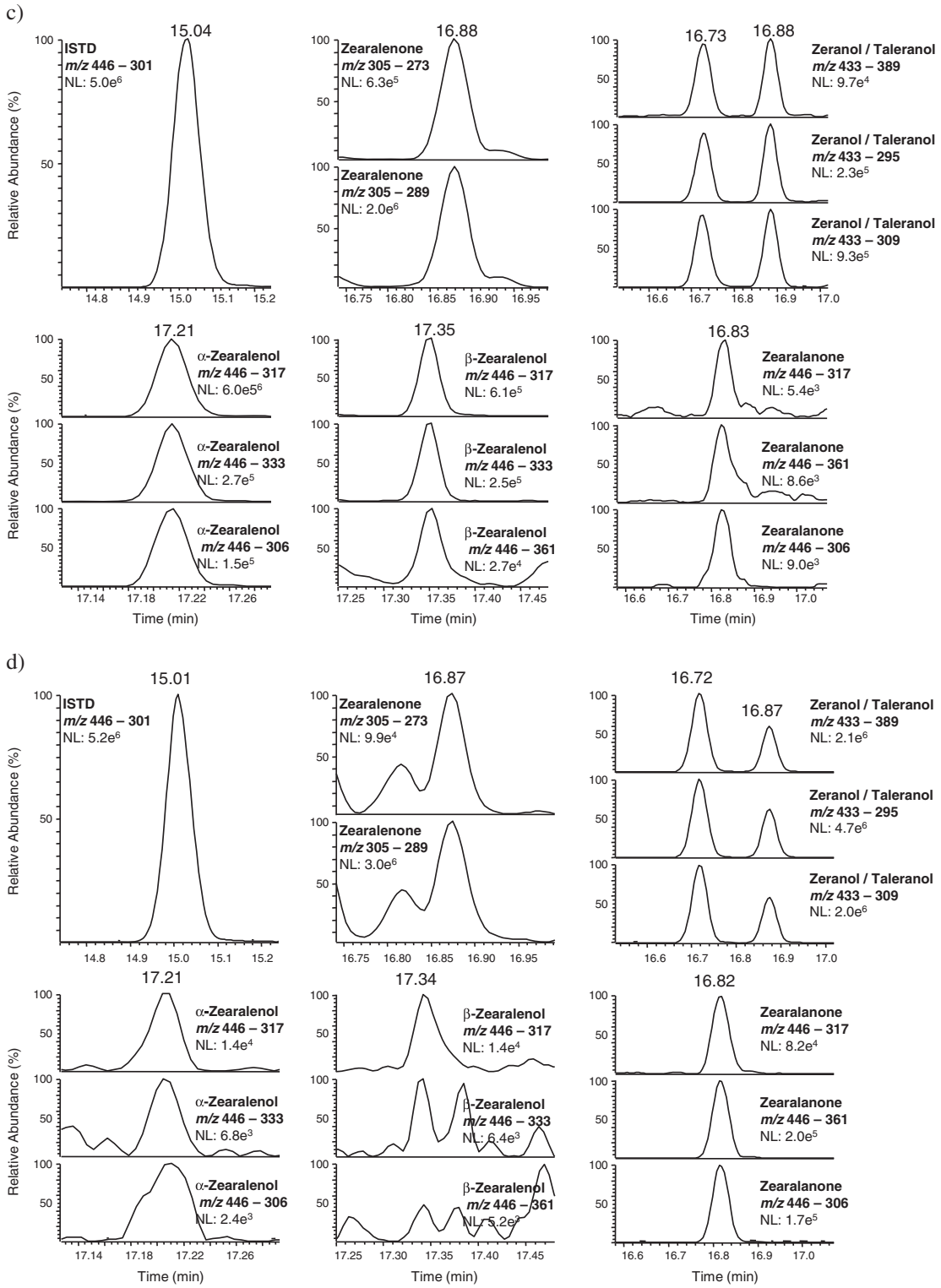

Figure 2. *(Continued)*

differences in the metabolic patterns (which might serve the purpose of potential decisive criteria) is the calculation of ratios as also suggested for veterinary and food safety purposes.[7] Particularly the ratio between α-zearalenol (**5**) and zeranol (**1**) was found to be of significance in cattle being >5 in case of mycotoxin ingestion. Applying this calculation to the doping control samples in question, ratios between 4.5 and 21 are obtained while the oral application of zeranol results in a ratio of 0.01 (Table 2).

The question whether the mycotoxins can be converted to zeranol (or its metabolites) and *vice versa* was qualitatively approached with *in vitro* simulation experiments using human

Table 2. Concentrations of target analytes estimated from doping control urine specimens (A–D) and an administration study urine sample (E). The ratio of α-zearalenol and zeranol might serve as discriminating criterion to differentiate mycotoxin contamination from zeranol application.

	Analyte concentration (ng/ml)						Ratio
Sample	zeranol (**1**)	taleranol (**3**)	zearalanone (**2**)	zearalenone (**4**)	α-zearalenol (**5**)	β-zearalenol (**6**)	α-zearalenol / zeranol (**5** / **1**)
A	1.3	1.5	0.9	6.1	8.4	2.1	6.5
B	1.0	0.9	1.0	4.4	4.5	0.7	4.5
C	2.3	1.5	0.5	23	28	6.3	12.2
D	1.4	1.3	0.5	23	30	7.3	21.0
E	27.8	17.8	19.5	0.2	0.2	n.d.	0.01

Table 3. Conversion of substrates to metabolites by *in vitro* experiments

Substrate/ /metabolite	zeranol (**1**)	taleranol (**3**)	zearalanone (**2**)	zearalenone (**4**)	α-zearalenol (**5**)	β-zearalenol (**6**)
zeranol (**1**)		++	+++	-	-	-
taleranol (**3**)	+		+++	-	-	-
zearalanone (**2**)	+++	++		-	-	-
zearalenone (**4**)	-	-	-		+++	++
α-zearalenol (**5**)	+	+	+	+++		+
β-zearalenol (**6**)	-	+	+	+++	++	

liver microsomal preparations. Each substrate (compounds **1–6**, Figure 1) was subjected to metabolizing enzymes and the resulting mixtures were analyzed by liquid chromatography - high resolution/high accuracy mass spectrometry. All substances were readily reduced and/or oxidized at C-7 to yield respective analogs, i.e. zeranol (**1**), taleranol (**3**), and zearalanone (**2**) were converted into each other and so were zearalenone (**4**), α- and β-zearalenol (**5** and **6**). The crossing of the metabolic pathways was, however, observed only from one direction allowing the C-2 – C-3 double bond to be reduced (e.g. in case of α-zearalenol) but not to be introduced (e.g. into zeranol) as summarized in Table 3. Although the methodology was aimed to be of qualitative nature only, a rough estimation of the conversion efficiency outlined only a low percentage of C-C double bond-reduced metabolites under the chosen *in vitro* conditions. These findings are in agreement with observations of earlier *in vivo* and *in vitro* studies conducted with different species[1,2,5,7,8,10,16–18] and are also in line with the analysis of the administration study urine specimen depicted in Figure 2d, where zeranol, taleranol, and zearalanone were detected almost exclusively.

Conclusions

The issue of mycotoxin contamination as a well-established phenomenon in food and veterinary control analyses might also apply to sports drug testing. According to reviews particularly considering the occurrence of zearalenone in foods and feeds – for example, the findings of infested wheat, barley, corn – has been reported for numerous countries of all continents in the last decade, and consequently, the ingestion of contaminated cereal products by athletes is a scenario to exclude.[8,14] The fact that no adverse analytical findings for zeranol were reported worldwide for several years substantiates the rarity of incidences where humans (rather than animals) are affected. Moreover, rather low urinary concentrations of zeranol as found in the doping control specimens might have contributed to a limited number of adverse analytical findings. Nevertheless, the possibility should be taken into account when zeranol is detected in sports drug testing samples, and the approach towards a differentiation between mycotoxin and anabolic agent administration by means of metabolite profiling seems adequate for doping control purposes also. Hence, suspicious initial testing results for zeranol should carefully be investigated concerning the presence or absence of derivatives being indicative for a zearalenone and/or zearalenol ingestion.

Acknowledgment

The presented study was conducted with support of the Manfred-Donike-Institute for Doping Analysis (Cologne, Germany).

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