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Insights into In Vivo Absolute Oral Bioavailability, Biotransformation, and Toxicokinetics of Zearalenone, α-Zearalenol, β-Zearalenol, Zearalenone-14-glucoside, and Zearalenone-14-sulfate in Pigs

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Supporting Information

ABSTRACT: The aim of this study was to determine the toxicokinetic characteristics of ZEN and its modified forms, α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalenone-14-glucoside (ZEN14G), and zearalenone-14-sulfate (ZEN14S), including presystemic and systemic hydrolysis in pigs. Crossover pig trials were performed by means of intravenous and oral administration of ZEN and its modified forms. Systemic plasma concentrations of the administered toxins and their metabolites were quantified and further processed via tailor-made compartmental toxicokinetic models. Furthermore, portal plasma was analyzed to unravel the site of hydrolysis, and urine samples were analyzed to determine urinary excretion. Results demonstrate complete presystemic hydrolysis of ZEN14G and ZEN14S to ZEN and high oral bioavailability for all administered compounds, with further extensive first-pass glucuronidation. Conclusively, the modified-ZEN forms α-ZEL, β-ZEL, ZEN14G, and ZEN14S contribute to overall ZEN systemic toxicity in pigs and should be taken into account for risk assessment.

KEYWORDS: *zearalenone, toxicokinetics, pig, zearalenone-14-glucoside, zearalenone-14-sulfate*

INTRODUCTION

Mycotoxins are known to be among the most hazardous of all food and feed contaminants in terms of chronic toxicity. Recent publications suggest that about 70% of cereal-based feeds are contaminated with one or more mycotoxins.[1]

Fusarium fungi frequently infect cereals (i.e., wheat, barley, rye, maize, and oats) in temperate regions, including Western Europe. The mycotoxins produced by these *Fusarium* species cause significant economic losses in animal production and are a hazard to public health and animal welfare. With an incidence of up to 80% in unprocessed cereals and up to nearly 100% in compound feed, zearalenone (ZEN) is one of the most critical *Fusarium* mycotoxins, because of its universal occurrence and toxic potential.[2,3] ZEN is a nonsteroidal mycoestrogen that binds to the 17β-estradiol receptor (ER) in target cells, leading to hyperestrogenism and reproductive disorders in both humans and pigs. Additionally, hemotoxic and genotoxic properties are reported.[4]

Aside from ZEN, modified-ZEN forms frequently coconta-minate cereal-based foods and feedstuff.[5−7] They are formed by plants and rival fungi by altering the chemical structure via biotransformation reactions as a natural protective strategy against xenobiotics. Important examples of these modified forms of ZEN are α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalenone-14-glucoside (ZEN14G), and zearalenone-14-sulfate (ZEN14S).[5,7,8] Although they exist in lower concentrations than the free or unconjugated forms, relative high incidence rates have been noted for modified mycotoxins in cereal-based food and feed matrices, with values of 53, 63, 30, and 20% for α-ZEL, β-ZEL, ZEN14G, and ZEN14S, respectively.[5] The same study suggests that approximately 60% of the available ZEN was found as modified forms.[6]

The susceptibility of humans and different animal species to a certain xenobiotic strongly depends on the oral bioavailability and biotransformation processes of that xenobiotic. For

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example, ZEN is efficiently absorbed after oral (PO) administration in pigs, with an absorbed fraction of 85%.[9] After oral absorption, its fate is determined by biotransformation, biliary excretion, and enterohepatic recycling (EHC).[9] As for the biotransformation, phase I reactions consist of reduction of ZEN to α-ZEL and β-ZEL, which is catalyzed by 3α- and 3β-hydroxysteroid dehydrogenases. Phase II biotransformation consists of glucuronidation of ZEN, α-ZEL, and β-ZEL, which is catalyzed by uridine diphosphate glucuronyl transferase (UDPGT).[10,11] Both biotransformation reactions mainly occur in the liver; however, in vitro experiments demonstrated the intestinal mucosa to be a possible extrahepatic biotransformation site.[9,12−15] Species that mainly metabolize ZEN to α-ZEL, such as humans and pigs, are relatively sensitive because of the higher affinity of α-ZEL for the ER, resulting in more potent toxic properties.[11,16−21] Curiously, Warth et al. did not detect α-ZEL in the urine of one human volunteer after ZEN administration.[21] In contrast, species that predominantly form β-ZEL, such as broiler chickens and rats, tend to be less sensitive because of the lower affinity of this metabolite for the ER.[11] More specifically, α-ZEL has a binding affinity to the ER 73 times higher than that of ZEN, whereas β-ZEL exhibits about half of the affinity that ZEN does.[22] Phase II metabolites have lost their affinity for the ER.[23,24] Nevertheless, cleavage (hydrolysis) during mammalian digestion of phase II modified forms produced by plants can release free ZEN.[25] Consequently, total exposures and risk assessments based only on free ZEN might be underestimated.

Hydrolysis of the phase II modified-ZEN forms mainly occurs in the gastrointestinal tract by enzymes of both the bacterial microbiota and the mammalian intestinal epithelium.[25] Hydrolysis of ZEN14G during digestion in pig was first demonstrated by Gareis et al.[26] An in vitro digestion study by Dall'Erta et al. showed that ZEN14G and ZEN14S are completely hydrolyzed to ZEN by the human colonic microbiota after an incubation time of only 30 min.[27] Besides enzymatic hydrolysis, acidic hydrolysis is also described. Glucosides seem to be more resistant to acid conditions in comparison with sulfate conjugates.[28] In contrast, an in vivo study in rats revealed hydrolysis of ZEN14G in the stomach.[29] Unfortunately, experiments studying the exact sites of hydrolysis are still lacking for ZEN conjugates. Sampling of portal plasma facilitates possible differentiation between presystemic (gastrointestinal) and systemic (hepatic, blood, etc.) hydrolysis of ZEN14G and ZEN14S, as previously described for deoxynivalenol-3-glucoside (DON3G).[30]

Following a request from the European Commission (EC), the European Food Safety Authority Panel on Contaminants in the Food Chain (EFSA CONTAM Panel) assessed whether it is appropriate and feasible to set a group-health-based guidance value for ZEN and its modified forms related to their presence in food and feed. The EFSA CONTAM Panel found it appropriate to set a group total daily intake (TDI) of 0.25 μg per kilogram of bodyweight (BW) per day, on the basis of a no-observed-effect level (NOEL) in gilts of 10 μg/kg BW, expressed as ZEN equivalents for ZEN and its modified forms. To take into account differences in estrogenic potency in vivo, each phase I metabolite was attributed a potency factor relative to ZEN to use for exposure estimates of the respective ZEN metabolites. Phase II metabolites of ZEN and of the phase I metabolites, which do not have estrogenic activity, were assumed to be cleaved, releasing ZEN and its phase I metabolites. These conjugates were attributed the same relative potency factors as the unconjugated forms. As such, relative potency factors of 60, 0.2, 1, and 1 were attributed to α-ZEL, β-ZEL, ZEN14G, and ZEN14S, respectively.[8]

Current potency factors were derived from rats, and until today, scientific data concerning the estrogenicity and absorption, distribution, metabolism, and excretion processes (ADME) of modified ZEN in humans and pigs are lacking.[31] Various assumptions and uncertainties are associated with the present risk assessment. The Panel recommended that more data on the toxicokinetics of the modified forms of ZEN were needed, particularly on their oral bioavailability and in vivo hydrolysis.[25,31,32]

Therefore, the goal of this study was to determine the absolute oral bioavailability and the main toxicokinetic parameters of α-ZEL, β-ZEL, ZEN14G, and ZEN14S, as well as the degree of presystemic and systemic hydrolysis of ZEN14G and ZEN14S in pigs, a species which, in addition to its susceptibility to the effects of ZEN, also provides a reliable model to extrapolate to humans.[33] In vivo models are preferred to in vitro models as these do not account for important physiological and anatomical factors, including intestinal mucosal- and luminal-content composition (enzymes and microbiota), internal-organ blood flow, and enterohepatic recirculation.[34]

MATERIALS AND METHODS

Standards, Reagents, and Solutions. ZEN, α-ZEL, and β-ZEL (>99% purity) were obtained from Fermentek (Jerusalem, Israel). α-Zearalanol (α-ZAL), β-zearalanol (β-ZAL), and zearalanone (ZAN) were purchased from Sigma-Aldrich (Bornem, Belgium). A $^{13}C_{18}$-ZEN (stable-isotope-labeled internal standard, IS; 25.4 μg/mL) stock solution in acetonitrile (ACN) was purchased from Biopure (Tulln, Austria). ZEN14G and ZEN14S were enzymatically synthesized, purified, and verified using nuclear magnetic resonance (NMR) and liquid chromatography−tandem mass spectrometry (LC-MS/MS).[35,36] No remaining ZEN (<0.05%) was detected in the produced ZEN14G and ZEN14S. The following characterized glucuronides (GlcA's) were available from earlier work: ZEN-14-*O*-glucuronide (ZEN-14-GlcA), α-ZEL-14-*O*-glucuronide (α-ZEL-14-GlcA), α-ZEL-7-*O*-glucuronide (α-ZEL-7-GlcA), β-ZEL-14-*O*-glucuronide (β-ZEL-14-GlcA), and β-ZEL-16-*O*-glucuronide (β-ZEL-16-GlcA).[37] ZEN, ZEN14G, α-ZEL, and β-ZEL were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Diegem, Belgium), providing a stock solution of 10 mg/mL to use for animal trials. ZEN, ZEN14G, α-ZEL, β-ZEL, α-ZAL, β-ZAL, and ZAN were dissolved in methanol (MeOH), providing a stock solution of 1 mg/mL to use for analytical experiments. ZEN14S was dissolved in water, providing a stock solution of 0.821 mg/mL. All GlcA's were dissolved in MeOH, yielding stock solutions of 50−500 μg/mL. Individual-standard working solutions of 50−100 μg/mL were obtained by diluting the stock solutions with ACN and were used for the analytical experiments. Standard-mixture working solutions were prepared by mixing appropriate dilutions of the individual-standard working solutions in ACN. For the IS, $^{13}C_{18}$-ZEN, a working solution of 100 ng/mL in ACN was prepared. All stock and working solutions were stored at less than or equal to −15 °C. Water, ACN, MeOH, and glacial acetic acid were of UHPLC-MS grade (Biosolve, Valkenswaard, The Netherlands).

Animal Trials. The study consisted of two animal trials. For each of the trials, eight clinically healthy pigs (sexes equally divided) were housed together in a suitable pen and supplied with feed and water ad libitum. The commercial pig feed was previously analyzed for the absence of mycotoxin contamination by LC-MS/MS. Temperature of the enclosure was controlled between 20 and 24 °C and natural light was implemented. After a 1 week acclimatization period, double lumen central venous catheters were surgically introduced via the vena

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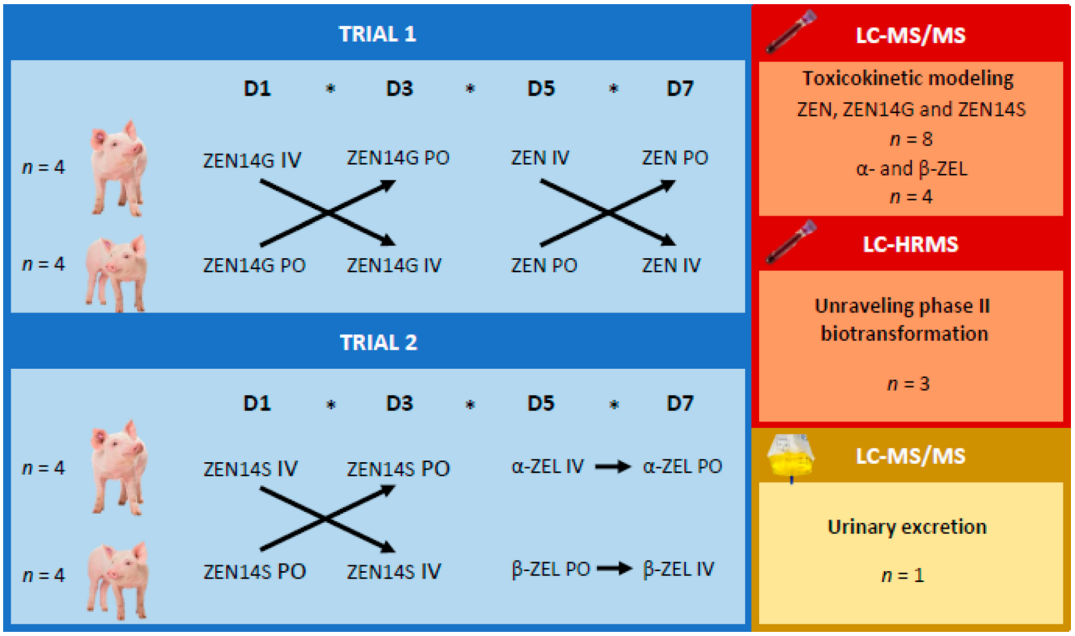

Figure 1. Experimental setup of the crossover animal trial investigating the toxicokinetics of zearalenone (ZEN) and its modified forms *α*-zearalenol (*α*-ZEL), *β*-zearalenol (*β*-ZEL), zearalenone-14-glucoside (ZEN14G), and zearalenone-14-sulfate (ZEN14S). *n*, number of animals per group; *D*, day of experiment; asterisk (*), wash-out period; IV, intravenously; PO, *per os*.

jugularis of each pig. In addition, four of the eight pigs were provided with a single lumen catheter introduced via the vena porta to study presystemic hydrolysis and biotransformation. The surgical procedure was performed as previously described by Gasthuys et al.[38] After surgery, all pigs were housed individually in order to prevent reciprocal mutilation and removal of the catheters. Figure 1 illustrates the experimental setup.

For the first trial, eight 8 week old pigs (Pietrain × Seghers hybrid, 16.5 ± 2.5 kg BW) were purchased from RA-SE Genetics (Lokeren, Belgium). A ZEN14G bolus (500 μg/kg BW) was administered to each animal by intravenous (IV) injection using the smallest lumen of the double lumen central venous catheters or orally (PO) by gastric gavage in a two-way crossover design. The dosage was based on a preliminary pilot study. After ZEN14G administration and a wash-out period of 48 h between treatments, identical crossover administration with ZEN (331 μg/kg BW, equimolar dose) was set up in the same animals. The animals were fasted starting 12 h before administration of the mycotoxins until 4 h postadministration (pa). The calculated amount of stock solution for each animal was diluted with ethanol to a volume of 1 mL (IV) and further diluted to 10 mL with water (PO). After PO dosing, the gavage tube was flushed with 50 mL of tap water. Blood (1−2 mL/sample) was sampled in EDTA tubes via the largest lumen of the central venous catheter and the vena porta catheter at 0 min (before administration) and at 5, 10, 20, 30, 45, 60, 90, 120, 240, 360, and 480 min pa. Blood samples were kept refrigerated (ice packs) and were centrifuged (2851*g*, 10 min, 4 °C) within 2 h after sampling. Plasma was then stored at ≤−15 °C until analysis. Simultaneously with blood collection, urine was sampled from one male piglet (chosen at random) at 4, 8, and 24 h pa for each of the administered toxins and administration routes (IV and PO). A noninvasive urine-sampling technique based on the application of urine pouches (only technically possible in boars) was used, as described by Gasthuys et al.[39] Urine samples were stored at ≤−15 °C until analysis.

For the second trial, eight 8 week old pigs (Pietrain × Seghers hybrid, 16.5 ± 2.3 kg BW) were purchased from RA-SE Genetics. First, each animals was administered a PO or IV ZEN14S bolus (415 μg/kg BW, equimolar dose), following a two-way crossover design, as described above. After a wash-out period of 48 h, the animals were treated with consecutive IV or PO boluses of either *α*-ZEL (333 μg/kg BW, equimolar dose, *n* = 4) or *β*-ZEL (333 μg/kg BW, equimolar dose, *n* = 4). All solvents and procedures were comparable to those used in the first trial.

The animal trials were approved by the Ethical Committee of the Faculties of Veterinary Medicine and Bioscience Engineering of Ghent University (EC2015/14). Care and use of animals was in full compliance with the most recent national legislation and European Directive.[40,41]

LC-MS/MS Analysis of Plasma. First, LC-MS/MS analysis of the plasma samples for ZEN, *α*-ZEL, *β*-ZEL, ZAN, *α*-ZAL, *β*-ZAL, ZEN14G, and ZEN14S was performed. Sample preparation and MS/MS settings were based on De Baere et al.[42] Compared with that method, ZEN14G and ZEN14S were also included in the current method. $^{13}C_{18}$-ZEN was used as the IS for all compounds. In brief, to 250 μL of plasma, 25 μL of the IS working solution was added.[42] After vortex mixing, ACN was added up to a volume of 1 mL to precipitate plasma proteins. Samples were then vortex-mixed again, and this was followed by centrifugation (8517*g*, 10 min, 4 °C). The supernatant was transferred to a new tube and evaporated to dryness under nitrogen at 45 ± 5 °C. The dry residue was then reconstituted in 200 μL of a mixture of water/MeOH (85:15, v/v) and microfiltrated (GV-PVDF 0.22 μm; Millipore, Overijse, Belgium), and 10 μL was injected onto the LC-MS/MS instrument. The LC-MS/MS system consisted of an Acquity UHPLC system coupled to a Xevo TQ-S MS instrument (Waters, Zellik, Belgium). Chromatographic separation of ZEN, *α*-ZEL, *β*-ZEL, ZEN14G, ZEN14S, ZAN, *α*-ZAL, and *β*-ZAL was achieved on an Acquity UPLC HSS T3 column (100 × 2.1 mm i.d., dp = 1.8 μm) in combination with an Acquity HSS T3 1.8 μm VanGuard precolumn, both of which were from Waters. All compounds were eluted with a gradient of water containing 0.01% glacial acetic acid (mobile phase A) and ACN (mobile phase B) at a flow rate of 300 μL/min. The following gradient-elution program was applied: 0−0.5 min (70% A, 30% B), 9 min (linear gradient to 70% B), 9−10.4 min (30% A, 70% B), 10.4−10.5 min (linear gradient to 70% A), 10.5−13 min (70% A, 30% B). MS/MS acquisition was performed in selected-reaction-monitoring (SRM) mode and negative-electrospray-ionization (ESI) mode. The mass-to-charge ratios (*m*/*z*) of the precursor ions were 479.1 and 397.0 for ZEN14G and ZEN14S, respectively. The product ions of ZEN14G and ZEN14S were *m*/*z* 175.0 and 317.1. Matrix-matched calibration curves (1/*x* weighted) and quality-control (QC) samples were prepared. The validation protocol and the acceptance criteria were

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according to De Baere et al.[45] and were based on VICH and EU guidelines.[43,44] The results of linearity, intraday apparent recovery and precision, interday apparent recovery and precision, limits of quantification (LOQs), and limits of detection (LODs) for ZEN, α-ZEL, β-ZEL, ZAN, α-ZAL, and β-ZAL are reported in De Baere et al.[42] The validation results for ZEN14G and ZEN14S can be found in Supplementary Table 1. LOQ's for the different analytes in the LC-MS/MS method ranged between 1 and 20 ng/mL. LC-MS/MS data acquisition and data processing were performed with Masslynx software (Waters).

LC-HRMS Analysis of Plasma. To determine the presence of phase II metabolites (i.e., glucuronides and possible sulfate conjugates) and to determine the type of glucuronide isomers formed, systemic plasma samples from three pigs for all bolus administrations were analyzed using validated ultrahigh-pressure liquid chromatography–high-resolution mass spectrometry (UPLC-HRMS). The following GlcA's were quantified: ZEN-14-GlcA, α-ZEL-14-GlcA, α-ZEL-7-GlcA, β-ZEL-14-GlcA, and β-ZEL-16-GlcA.

Briefly, 150 μL of plasma for each sample was mixed with 450 μL of ACN and vortexed for 30 s. After protein precipitation by centrifugation (20 000*g*, 10 min, 4 °C), the supernatant (450 μL) was transferred into a 10 mL conical glass tube and evaporated to dryness under nitrogen at 60 °C. The dry residue was reconstituted in 100 μL of a mixture of ACN/water (50:50, v/v) and microfiltrated (Costar Spin-X 0.22 mm Nylon filter; Corning, Inc., Corning, NY), and 5 μL was injected onto the LC-HRMS instrument. The LC-HRMS instrument consisted of a QExactive Hybrid Quadrupole-Orbitrap mass spectrometer equipped with a heated electrospray-ionization source (HESI-II) and coupled to a Vanquish UHPLC system (Thermo Fisher Scientific, Waltham, MA). The mobile phase consisted of water (A) or ACN (B) containing 0.2% formic acid at a flow rate of 250 μL/min. The gradient separation was achieved starting from 40% B at 1 min and increasing to 97% B at 27 min. A representative chromatogram is shown in Supplementary Figure 1. The HESI-II interface was operated at 300 °C in negative- and positive-ionization modes using fast polarity switching in the mass range of m/z 200–720. Chromatographic separation was performed at 30 °C on a 150 × 2.1 mm i.d. Kinetex F5 LC column (2.6 μm; Phenomenex, Utrecht, The Netherlands) with a 0.5 μm × 0.004 in. i.d. HPLC KrudKatcher Ultra Column In-Line filter (Phenomenex, Utrecht, The Netherlands). The identification of ZEN, α-ZEL, β-ZEL, α-ZAL, β-ZAL, ZEN-14-GlcA, α-ZEL-14-GlcA, α-ZEL-7-GlcA, β-ZEL-14-GlcA, and β-ZEL-16-GlcA was confirmed by comparison to authentic reference standards. The verification procedure was based on compound-specific retention times, fragmentation patterns and accurate masses, which were obtained using a mass accuracy window of ±5 ppm with respect to the theoretical accurate masses. Xcalibur 2.2 software (Thermo Fisher Scientific) was used for instrument control and data evaluation. The molecular formulas and exact masses of the target compounds were calculated using the built-in Qualbrowser of the Xcalibur 2.2 software.

Unweighted matrix-assisted calibrations as well as calibrations prepared in ACN/water (50:50, v/v) were used, employing external-standard procedures and ranging from 1 to 200 ng/mL for all analytes included in this study. LOQ's for the different analytes in the LC-HRMS method ranged between 3.7 and 10.2 ng/mL. The results of the linearity, apparent recovery, standard error of the mean, LOD, LOQ, signal suppression–enhancement and total relative standard deviation of the LC-HRMS method validation can be found in Supplementary Table 2.

LC-MS/MS Analysis of Urine. In order to verify whether urinary excretion of ZEN and its metabolites corresponds with results in plasma, urine of one random male pig for each toxin and each administration route (IV and PO) was analyzed by a validated LC-MS/MS method.

Briefly, 4 g of sample was mixed with 20 mL of ACN acidified with formic acid (99:1, v/v), and the sample was agitated for 60 min on a Reax 2 overhead shaker (VWR, Haasrode, Belgium). After addition of 4 g of $MgSO_4$ and 1 g of NaCl, the sample was shaken and centrifuged (16 800*g*, 5 min). The upper organic layer (4 mL) was microfiltered through a 0.2 μm RC filter and transferred to a glass evaporation tube. Monoethylene glycol (10 μL) was added as a keeper solvent, and this solution was evaporated to dryness under nitrogen at 40 °C. The dry residue was reconstituted in 1 mL of an aqueous methanolic solution (MeOH/water, 80:20, v/v) and mixed for 30 s, and then 5 μL was injected into the chromatographic system. The LC system consisted of an Acquity UPLC H-Class (Waters) equipped with a quaternary solvent manager and a flow-through needle sample manager. The analytical column used was a Waters Acquity UPLC HSS T3 column (100 × 2.1 mm i.d., dp = 1.8 μm) kept at 40 °C; this was preceded by a Waters Acquity UPLC BEH C18 VanGuard precolumn (5 × 2.1 mm i.d., dp = 1.7 μm). All compounds were eluted with a gradient of water containing ammonium acetate (5 mmol/L) and acetic acid (0.05 vol %, mobile phase A) and MeOH containing ammonium acetate (5 mmol/L) and acetic acid (0.05 vol %, mobile phase B) at a flow rate of 500 μL/min. The gradient program started at 2.5% B and linearly increased after 1 min to 99% B in 15 min. The column was washed with 99% MeOH for 1 min and equilibrated at initial conditions for 4 min, resulting in a total run time of 20 min. A Xevo TQ-S mass spectrometer (Waters), equipped with an ESI source, was used as a detector and operated with Masslynx software (Waters). MS/MS acquisition was performed in SRM mode. ZEN, α-ZEL, β-ZEL, ZEN14S, and ZEN14G were measured in negative-ionization mode, whereas ZEN-14-GlcA, α-ZEL-14-GlcA, and β-ZEL-14-GlcA were measured in positive-ionization mode. The source temperature was set at 150 °C, and the capillary desolvation heater was set at 450 °C. The capillary voltage used was 0.5 kV, and nitrogen was used as the drying gas at a flow rate of 1000 L/min in both positive- and negative-ion mode. A five-point, $1/x$-weighted calibration curve (water/MeOH, 50:50, v/v) was used (range 0.5–50 ng/mL). After 10 injections, a standard working solution of 10 ng/mL was injected in order to verify the retention time. Compound identification was based on retention times (±2.5%), and ion ratios were defined as qualifier-ion peak area/quantifier-ion peak area. The acceptance criteria were ±20% if the ratio was between 0.5 and 1 and ±25% if it was lower than 0.5. The results of the linearity, intraday precision, interday apparent recovery and precision, LOD, and LOQ can be found in Supplementary Table 3.

Toxicokinetic Modeling. Toxicokinetic modeling was performed on the basis of the LC-MS/MS data in plasma, using Phoenix6.4 software (Certara, Cary, NC). Plasma concentrations below the LOQ were not taken into account. Therefore, none of the PO data could be included. Molar concentrations, expressed as nanomoles per milliliter (nmol/mL), were used for the toxicokinetic analysis. Multiplicative weighing was applied for all calculations.

For the ZEN, α-ZEL, and β-ZEL IV data, tailor-made one-compartment models with first-order elimination were applied.

The estimated primary parameters were the volume of distribution (Vd), the clearance (CL), the clearance of ZEN to α-ZEL ($CL_{ZEN\rightarrow ZEL}$), and the clearance of α-ZEL after ZEN administration ($CL_{\alpha\text{-ZEL}}$). The Vd of α-ZEL after IV ZEN administration was fixed to the average Vd of α-ZEL obtained after IV α-ZEL administration. Secondary parameters were the area under the plasma-concentration-time curve from time zero to infinity ($AUC_{0-\infty}$), the elimination rate constant (k_e), and the elimination half-life ($t_{1/2el}$). Estimated secondary parameters of α-ZEL after IV ZEN administration were $k_{e,\alpha\text{-ZEL}}$ and $t_{1/2el,\alpha\text{-ZEL}}$. The percentage of conversion (CONV) of ZEN to α-ZEL (and vice versa) was calculated as follows:

$$\mathrm{CONV}_{\mathrm{ZEN}\rightarrow\alpha\text{-ZEL}}\ (\%) = \frac{\mathrm{AUC}_{\alpha\text{-ZEL(post-ZEN-administration)}} \times \mathrm{CL}_{\alpha\text{-ZEL}}}{\mathrm{AUC}_{\mathrm{ZEN(postadministration)}} \times \mathrm{CL}_{\mathrm{ZEN}}} \times 100 \quad (1)$$

$$\mathrm{CONV}_{\alpha\text{-ZEL}\rightarrow\mathrm{ZEN}}\ (\%) = \frac{\mathrm{AUC}_{\mathrm{ZEN(post-}\alpha\text{-ZEL-administration)}} \times \mathrm{CL}_{\mathrm{ZEN}}}{\mathrm{AUC}_{\alpha\text{-ZEL(post-}\alpha\text{-ZEL-administration)}} \times \mathrm{CL}_{\alpha\text{-ZEL}}} \times 100 \quad (2)$$

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For IV ZEN14G analysis, a tailor-made two-compartmental model with first-order elimination was applied. A graphical representation of the applied model is given in Figure 2. Again, the Vd and CL of ZEN

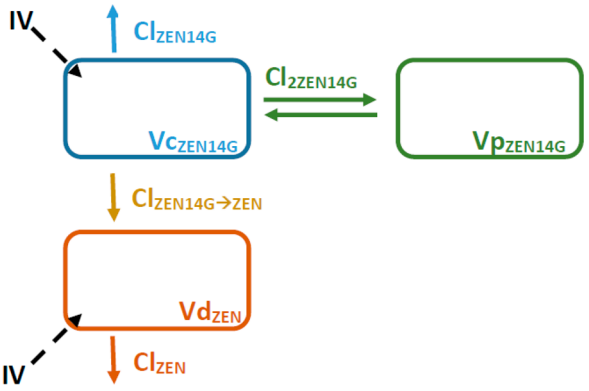

Figure 2. Graphical representation of the applied model for ZEN14G after intravenous (IV) administration: $V_{cZEN14G}$, volume of distribution of the central compartment for ZEN14G (L/kg); $V_{pZEN14G}$, volume of distribution of the peripheral compartment for ZEN14G (L/kg); V_{dZEN}, volume of distribution for ZEN (L/kg); CL_{ZEN14G}, clearance of ZEN14G (L/kg·h); $CL_{2ZEN14G}$, intercompartmental flow for ZEN14G (L/kg·h); $CL_{ZEN14G\rightarrow ZEN}$, clearance of ZEN14G by systemic hydrolysis to ZEN; CL_{ZEN}, clearance of ZEN after ZEN14G administration (L/kg·h).

after IV ZEN14G administration were fixed to the average Vd and CL of ZEN obtained after IV ZEN administration. The estimated primary parameters of ZEN14G were the Vd of the central compartment (Vc/VcZEN14G) and peripheral compartment (Vp/VpZEN14G), the clearance from the central compartment (CL), the intercompartmental flow ($CL_{2ZEN14G}$), and the clearance of ZEN14G to ZEN ($CL_{ZEN14G\rightarrow ZEN}$). The secondary parameters were $AUC_{0-\infty}$, k_e, and $t_{1/2el}$. The estimated secondary parameters of ZEN after IV ZEN14G administration were $k_{e,ZEN}$ and $t_{1/2el,ZEN}$. The percentage of systemic hydrolysis (SH) of ZEN14G to ZEN was calculated according to the following formula:

$$\begin{aligned} SH\ (\%) = \{&[AUC_{ZEN} \times CL_{ZEN}]/[AUC_{ZEN14G} \\ &\times (CL_{ZEN14G} + CL_{ZEN14G\rightarrow ZEN}) + AUC_{ZEN} \\ &\times CL_{ZEN}]\} \times 100 \end{aligned} \quad (3)$$

After IV ZEN14S administration, no plasma concentrations could be detected via LC-MS/MS, and consequently, no further toxicokinetic modeling could be performed.

Although no toxicokinetic modeling could be performed on the LC-MS/MS data obtained after PO administration of the toxins, some estimates could be calculated on the basis of the LC-MS/MS and LC-HRMS data. These are only estimates because the results are based on data from only three pigs. An equimolar correction was applied for all values. Absolute oral bioavailability (F, i.e., the fraction of the administered toxin that is absorbed in systemic circulation in its unchanged form) could be estimated as follows:

$$F\ (\%) \approx \frac{AUC_{PO(toxin+metabolites)}}{AUC_{IV(toxin+metabolites)}} \times 100 \quad (4)$$

For phase II modified mycotoxins, the absorbed fraction (FRAC, i.e., the fraction of the administered toxin that is absorbed in systemic circulation in its unchanged form and in its hydrolyzed form) could be estimated as follows:

$$FRAC\ (\%) \approx \frac{AUC_{PO[toxin(administered)+toxin(hydrolyzed)+metabolites]}}{AUC_{IV[toxin(administered)+toxin(hydrolyzed)+metabolites]}} \times 100 \quad (5)$$

RESULTS AND DISCUSSION

Systemic Plasma Analysis and Toxicokinetic Analysis.

The aim of this study was to determine the absolute oral bioavailability of ZEN, α-ZEL, β-ZEL, ZEN14G, and ZEN14S and their biotransformations; the degree of in vivo hydrolysis of ZEN14G and ZEN14S to ZEN (differentiating between presystemic and systemic hydrolysis); and the toxicokinetic parameters of different ZEN forms in pigs. Tailor-made compartmental toxicokinetic models were developed, which offered the advantage, compared with noncompartmental analysis, of allowing us to take into account metabolites as well as make predictions about concentrations at unsampled time points.[46]

Aside from edematous swelling of the vulva, no adverse effects were detected during the animal trials following all bolus administration.

Plasma-concentration-time profiles, obtained after LC-MS/MS and LC-HRMS analysis, for all bolus administrations are presented in Figure 3. Each profile represents the mean plus the standard deviation (SD) of n animals for systemic plasma concentrations (n can deviate because of problems during blood sampling due to catheter occlusions). Values below the LOQ were not included. No ZAN, α-ZAL, or β-ZAL were detected after IV and PO administration of ZEN, α-ZEL, β-ZEL, ZEN14G, and ZEN14S, and this is in accordance with previously published data.[42,47]

After oral administration, plasma concentrations of ZEN, its modified forms, and its phase I metabolites, were too often below the LOQ to construct reliable plasma-concentration-time profiles. This can be attributed to the extensive first-pass biotransformation of ZEN and its modified forms. Pfeiffer et al. determined the in vitro glucuronidation activities for ZEN and its phase I metabolites using hepatic microsomes from various farm animals, experimental animals, and humans, showing the highest activity for porcine microsomes.[14] Indeed, fast and extensive glucuronidation of ZEN, α-ZEL, and β-ZEL was detected after PO administration.

After IV ZEN administration, a partial phase I biotransformation of ZEN to α-ZEL but not to β-ZEL was observed. This conversion was estimated to be approximately 60% on the basis of the area under the curve ($AUC_{0-\infty}$) corrected for differences in the CL values of both ZEN and α-ZEL. These findings are similar to the data obtained by Fleck et al. after IV ZEN administration (0.1 mg/kg BW).[47] Some controversy exists concerning β-ZEL as a ZEN metabolite in pig. α-ZEL is generally considered the main phase I metabolite in pig. Nevertheless, some studies report the presence of β-ZEL and β-ZEL-GlcA in pig urine after oral administration of ZEN, with α/β ratios ranging from 3 up to 36.5.[10,47−51] Differences in α/β-ZEL ratios could be explained by genetic variation within the different pig breeds or even individuals. After all, in vitro studies have demonstrated porcine-hepatic-microsomal reduction of ZEN to both α- and β-ZEL.[52−54] The derived main toxicokinetic parameters after IV administration of ZEN, α-ZEL, β-ZEL, and ZEN14G are shown in Table 1.

Within multiple in vivo pig studies concerning the toxicokinetics of ZEN, the elimination half-life of ZEN showed high variation (2.63−86.6 h).[9,47,50,55−57] These values are higher than the elimination half-life of ZEN ($t_{1/2el,ZEN}$ = 0.95 ± 0.45 h) calculated in the current study. This discrepancy can be explained by the fact that the other studies took into account the GlcA's, which show a longer persistence in biological matrices such as plasma and urine. Only one in vivo

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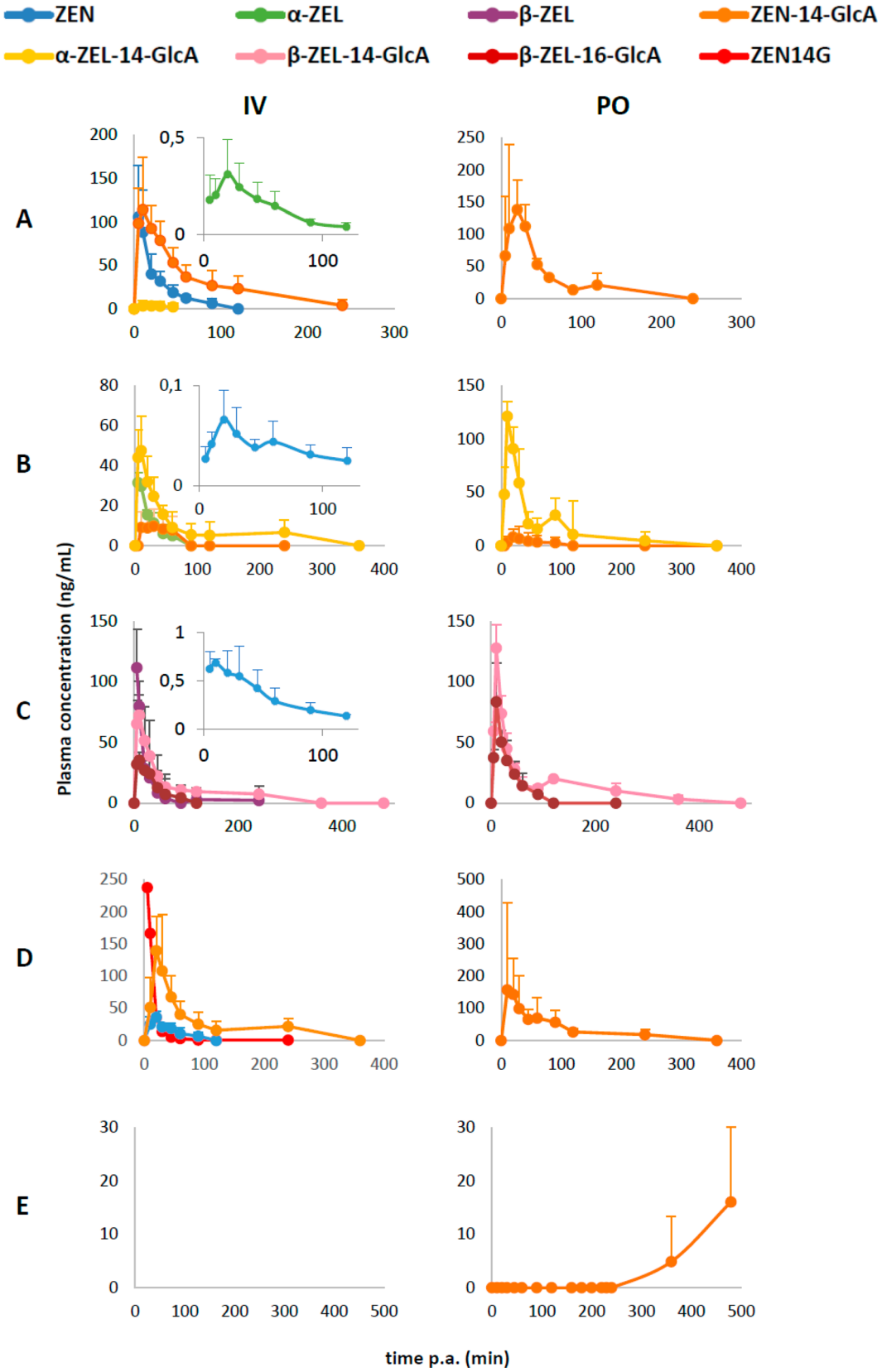

Figure 3. Systemic plasma-concentration-time profiles after oral (PO) and intravenous (IV) administration of (A) zearalenone (ZEN, dose of 331 μg/kg BW, n = 5), (B) α-zearalenol (α-ZEL, 333 μg/kg BW, n = 3), (C) β-zearalenol (β-ZEL, 333 μg/kg BW, n = 3), (D) zearalenone-14-glucoside (ZEN14G, 500 μg/kg BW, n = 7), and (E) zearalenone-14-sulfate (ZEN14S, 415 μg/kg BW, n = 3) to pigs. Values are presented as means + standard deviations. Plasma concentrations of ZEN, α-ZEL, β-ZEL, ZEN14G, and ZEN14S were quantified using LC-MS/MS. Plasma concentrations of phase II glucuronide metabolites were quantified using LC-HRMS (n = 3). After IV ZEN14S administration, none of the analytes could be detected (<LOQ).

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Table 1. Toxicokinetic Parameters after Intravenous (IV) and Oral (PO) Administration of Zearalenone (ZEN),[a] α-Zearalenol (α-ZEL),[b] β-Zearalenol (β-ZEL),[b] and Zearalenone-14-glucoside (ZEN14G)[c] to Pigs[d]

intravenous administration				
	ZEN	α-ZEL	β-ZEL	ZEN14G
CL (L/kg·h)	6.66 ± 2.74	62.47 ± 17.72	4.23 ± 0.09	3.67 ± 1.80
CL_{ZEN}	N/A	N/A	N/A	10.15 ± 2.14
$CL_{ZEN→ZEL}$	6.18 ± 2.75	N/A	N/A	N/A
$CL_{α\text{-}ZEL}$	41.05 ± 19.83	N/A	N/A	N/A
$CL_{2ZEN14G}$	N/A	N/A	N/A	1.06 ± 0.22
$CL_{ZEN14G→ZEN}$	N/A	N/A	N/A	2.98 ± 0.60
Vd (L/kg)	7.27 ± 0.78	8.47 ± 0.41	1.56 ± 0.04	N/A
Vc / VcZEN14G	N/A	N/A	N/A	0.57 ± 0.33
Vp / VpZEN14G	N/A	N/A	N/A	0.41 ± 0.14
C_0 (nmol/mL)	0.15 ± 0.01	0.12 ± 0.006	0.67 ± 0.02	2.64 ± 1.19
$AUC_{0-∞}$ (nmol·h/mL)	0.19 ± 0.08	0.018 ± 0.005	0.246 ± 0.005	0.25 ± 0.06
k_e (h^{-1})	0.95 ± 0.45	7.50 ± 2.46	2.72 ± 0.12	8.19 ± 2.60
$k_{e,α\text{-}ZEL}$	4.83 ± 2.33	N/A	N/A	N/A
$k_{e,ZEN}$	N/A	N/A	N/A	1.52 ± 0.32
$t_{1/2el}$ (h)	0.95 ± 0.45	0.10 ± 0.03	0.26 ± 0.01	0.10 ± 0.03
$t_{1/2el,α\text{-}ZEL}$	0.18 ± 0.06	N/A	N/A	N/A
$t_{1/2el,ZEN}$	N/A	N/A	N/A	0.49 ± 0.12
SH (%)	N/A	N/A	N/A	19.95 ± 1.59
oral administration				
	ZEN	α-ZEL	β-ZEL	ZEN14G
F (%)	61	123	98	N/A
FRAC (%)	N/A	N/A	N/A	61
PH (%)	N/A	N/A	N/A	100

[a]IV: n = 5, PO: n = 3. [b]n = 3. [c]IV: n = 7, PO: n = 3. [d]Values are presented as means ± standard deviations. CL, clearance of the administered mycotoxin (L/kg·h); CL_{ZEN}, clearance of ZEN after ZEN14G administration (L/kg·h); $CL_{ZEN→ZEL}$, clearance of ZEN by biotransformation to ZEL (L/kg·h); $CL_{α\text{-}ZEL}$, clearance of α-ZEL after ZEN administration (L/kg·h); $CL_{2ZEN14G}$, intercompartmental flow for ZEN14G (L/kg·h); $CL_{ZEN14G→ZEN}$, clearance of ZEN14G by systemic hydrolysis to ZEN; V_d, volume of distribution for the administered mycotoxin (L/kg); $V_{c,ZEN14G}$, central volume of distribution for ZEN14G (L/kg); $V_{p,ZEN14G}$, peripheral volume of distribution for ZEN14G (L/kg); C_0, plasma concentration of the administered mycotoxin at the time of administration (nmol/mL); $AUC_{0-∞}$, area under the plasma-concentration-time curve from the time of administration (0) to infinity (nmol·h/mL); k_e, elimination rate constant for the administered mycotoxin (h^{-1}); $k_{e,α\text{-}ZEL}$, elimination rate constant for α-ZEL after ZEN administration (h^{-1}); $k_{e,ZEN}$, elimination rate constant for ZEN after ZEN14G administration (h^{-1}); $t_{1/2el}$, elimination half-life of the administered mycotoxin (h); $t_{1/2el,α\text{-}ZEL}$, elimination half-life of α-ZEL after ZEN administration (h); $t_{1/2el,ZEN}$, elimination half-life of ZEN after ZEN14G administration (h); SH, percentage of the administered fraction of ZEN14G that is systemically hydrolyzed to ZEN (%); *F*, oral bioavailability (%); FRAC, absorbed fraction (%); N/A, not applicable; PH, presystemic hydrolysis.

study was performed in which toxicokinetic parameters of free ZEN were calculated after IV administration of ZEN to pigs.[47] In that study, IV injection of 0.1 mg/kg BW ZEN resulted in rapid distribution from the plasma ($t_{1/2}$ = 0.3−1 h). This is comparable with the elimination half-life of ZEN obtained in the present study. Additionally, similar CL rates were observed in both studies, with values of 5.1 ± 1.4 and 6.66 ± 2.74 L/kg·h in the study of Fleck et al. and in present study, respectively.[47]

After both IV α-ZEL and β-ZEL administration, partial conversion to ZEN was demonstrated. Using a similar calculation approach as for the conversion of ZEN to α-ZEL, the conversion of α-ZEL to ZEN was estimated to be approximately 7% after IV α-ZEL administration. No conversion between α-ZEL and β-ZEL was observed.

After IV ZEN14G administration, ZEN14G undergoes partial hydrolysis to ZEN (19.95 ± 1.59%). Systemic hydrolysis can be attributed to the effects of liver enzymes and blood esterase enzymes. This hydrolysis of ZEN14G is in contrast to the observations for DON3G by Broekaert et al., where no systemic hydrolysis was detected of DON3G after IV administration to pigs.[30] However, Broekaert et al. did describe the presence of systemic hydrolysis for the 3- and 15-acetylated forms of deoxynivalenol.

In remarkable contrast to ZEN14G, no ZEN14S, ZEN, or ZEL was detected after IV ZEN14S administration with either LC-MS/MS or LC-HRMS. This indicates that ZEN14S is not systemically hydrolyzed but assumably is rapidly cleared from the system, thereby swiftly reducing plasma concentrations of ZEN14S below the relatively high LOQ (20 ng/mL).

The elimination half-lives of the modified-ZEN forms appeared to be smaller than that of ZEN itself, caused by either higher CL values (α-ZEL) or lower volumes of distribution (β-ZEL and ZEN14G), due to their higher polarities compared with that of ZEN. Another explanation could possibly be differences in plasma-protein binding.

In contrast to ZEN and its phase I metabolites, the GlcA metabolites of ZEN, α-ZEL, and β-ZEL could be detected after PO administration, demonstrating systemic exposure to all of the orally administered ZEN forms and demonstrating the potential of these phase II metabolites as biomarkers for ZEN exposure in pigs. This is in line with the studies of Binder et al. and Fleck et al., in which phase II glucuronidation of ZEN was described.[47,50] Besides pigs, ZEN-glucuronide (ZEN-GlcA) has additionally been demonstrated to be the main urinary-

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excretion product after oral administration of ZEN to one human volunteer.[21] Because of its two hydroxyl groups (i.e., at positions 14 and 16), ZEN can theoretically form two regio-isomeric monoglucuronides. Results for ZEN- and ZEL-GlcA's revealed that exclusively the 14-isomers (i.e., ZEN-14-GlcA and α-ZEL-14-GlcA isomers) were formed after ZEN, α-ZEL, ZEN14G, and ZEN14S bolus administration. This is in accordance with Pfeiffer et al., who incubated ZEN with hepatic microsomes from different species in the presence of uridine-5′-diphosphoglucuronic acid (UDPGA). ZEN-14-GlcA was the predominant glucuronide in all species.[14] Results after β-ZEL bolus administration showed that both β-ZEL-14-glucuronide and β-ZEL-16-glucuronide were formed. In contrast to the results of Ueberschär et al., no sulfate conjugates of α-ZEL were observed.[58] Curiously, Ueberschär described 62% of the total dose of ZEN metabolites recovered in urine to be in the sulfated form, although pigs are generally known to have a low phase II sulfation activity.[58−61]

On the basis of LC-MS/MS and LC-HRMS data, estimated oral bioavailability for each of the administered toxins (except for ZEN14S) could be calculated (see Table 1). For ZEN, this results in an estimated *F* of 61%, which is comparable to the findings of Fleck et al., where an *F* of 59% was calculated for pigs of the same age.[47] In a study in older pigs (10−14 weeks old) a higher *F* of 80−85% was found.[9] For α-ZEL, an estimated *F* of 123% was calculated. Evidently, this value is an overestimation, as no correction for clearance could be applied and as Table 1 shows a relatively high clearance of α-ZEL. For β-ZEL the estimated *F* was 98%. These high values for α- and β-ZEL could indicate that ZELs are more easily absorbed than ZEN itself, approaching complete oral absorption.

For ZEN14G this resulted in an estimated FRAC of 61%, suggesting complete hydrolysis of ZEN14G to ZEN after PO administration.

After PO ZEN14S administration, a lag time of over 4 h pa can be observed, before quantifiable plasma concentrations of ZEN-14-GlcA were detected. Because of this lag time, the plasma-concentration-time profile for the ZEN14S metabolites exceeds the sampling period of 480 min, resulting in an incomplete profile. This lag time suggests hydrolysis of ZEN14S occurs distally in the gastrointestinal tract, presumably in the colon by microflora, which is in accordance with a study published by Dall'Erta et al. on in vitro digestion, which showed that ZEN14S is rapidly deconjugated to ZEN by colonic microbiota in a simulation of the large intestines.[27] This is in contrast to the results after oral ZEN14G administration, where no lag time was observed, suggesting hydrolysis occurs proximally at the main site of absorption (duodenum and proximal jejunum), presumable by the acidic conditions in the stomach and duodenum or by enzymes of the intestinal epithelium or microbiota.[62] Proximal gastrointestinal hydrolysis of ZEN14G is supported by equal FRAC and *F* values for ZEN14G and ZEN. Because of the incomplete profile for ZEN14S, no estimates for FRAC could be made, although presystemic (gastrointestinal) hydrolysis of ZEN14S is clearly present. This is in accordance with previous studies, both in pig and after exposure to human intestinal microflora.[27,50]

After all PO administrations, the profiles of the GlcA's show a second peak in the plasma-concentration-time profiles during the elimination phase, at about 2 h pa. This phenomenon can possibly be attributed to the EHC processes and has previously been described by several authors.[9,63]

Portal-Plasma Analysis. Additionally, portal-plasma samples were analyzed after all oral bolus administrations (Supplementary Figure 2). No ZEN14G or ZEN14S could be detected in portal plasma, supporting the hypothesis that hydrolysis of the modified-ZEN forms to ZEN must occur before portal plasma is reached (i.e., in the gastrointestinal tract both by enzymes of the bacterial microbiota and the mammalian intestinal epithelium and by the acidic conditions of the stomach).[25−27] After PO α-ZEL and β-ZEL administration, low concentrations of α-ZEL and β-ZEL, respectively, could be detected in the portal plasma and not in the jugular plasma, suggesting these phase I modified mycotoxins are intestinally absorbed as such and then undergo extensive first-pass hepatic glucuronidation. Unfortunately, this could not be confirmed for ZEN, assumably because of the lower *F* for ZEN in comparison with those of α- and β-ZEL and consequently the lower plasma concentrations of ZEN (<LOQ).

LC-MS/MS Analysis of Urine. Figure 4 displays the concentrations of mycotoxins recovered in the urine of a representative pig at 4, 8, and 24 h after PO and IV ZEN14G administration. The absence of ZEN14G in urine after PO administration corresponds with the plasma results and with the findings of Binder et al. and Dall'Erta et al.[27,50] No ZEN14G in urine was detected after IV administration as well. This is in contrast with results after plasma analysis and could hypothetically be caused by autohydrolysis of ZEN14G to ZEN, explaining the relatively high concentrations of ZEN. In accordance with the results of plasma analysis after all bolus administrations, the administered toxins are mainly recovered as their GlcA metabolites (Supplementary Figure 3). Hence, on the basis of the excretion of the toxins in urine and possible autohydrolysis of GlcA's, ZEN and its phase I metabolites could be recovered after oral administration in urine.

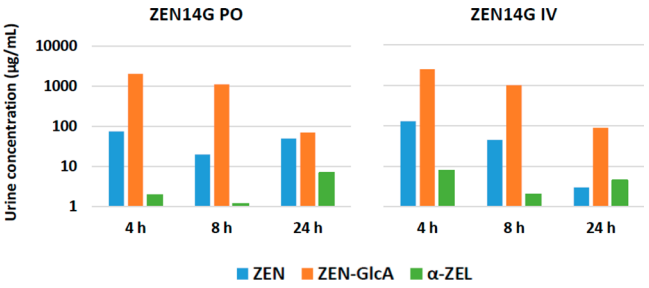

Figure 4. Urine concentrations (μg/mL, log-scaled) of zearalenone (ZEN) and its metabolites recovered in urine of a representative pig at 4, 8, and 24 h after PO and IV administration of zearalenone-14-glucoside (ZEN14G, 500 μg/kg BW). α-ZEL, α-zearalenol; ZEN-GlcA, zearalenone-14-glucuronide.

In conclusion, on the basis of the plasma results obtained in this study, an estimated oral bioavailability for ZEN of 61% was found, in accordance with previous results in pigs of the same age.[47] In older pigs, higher oral bioavailability (80−85%) was observed, pointing toward possible age-related differences in the toxicokinetics of ZEN and its modified forms.[9] α- and β-ZEL were completely absorbed after oral administration. For ZEN14G, an absorbed fraction of 61% was estimated, suggesting complete presystemic (proximal gastrointestinal) hydrolysis of ZEN14G to ZEN after PO administration and absorption as such. After both PO and IV administration of all ZEN forms, extensive phase II glucuronidation seems to occur. Hence, α-ZEL, β-ZEL, ZEN14G, and ZEN14S contribute to

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total systemic ZEN toxicity. These results highlight the importance of including modified-ZEN forms in feed analysis and GlcA metabolites in biomonitoring studies and diagnosis. Additionally, results suggest that further research concerning age-related differences in the toxicokinetics of ZEN and its modified forms should be performed. Supported by the anatomical and physiological similarities between pigs and human, these data may contribute in unraveling some of the uncertainties associated with the present risk assessment concerning the absolute oral bioavailability, biotransformation, and toxicokinetics of modified-ZEN forms.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b05838.

Representative LC-HRMS extracted-ion chromatograms corresponding to the deprotonated ($[M-H]^-$) ions of ZEN (m/z 317.1394), ZEN-GlcA (m/z 493.1715), $\alpha(\beta)$-ZEL (m/z 319.1551), $\alpha(\beta)$-ZAL (m/z 321.1707), and $\alpha(\beta)$-ZEL-GlcA (m/z 495.1872); portal-plasma-concentration-time profiles (LC-HRMS) after oral administration of zearalenone, α-zearalenol, β-zearalenol, zearalenone-14-glucoside, and zearalenone-14-sulfate to one pig; urine concentrations of ZEN and its metabolites recovered in the urine of a representative pig at 4, 8, and 24 h after PO and IV administration of ZEN, α-zearalenol, and β-zearalenol; results of the linearity ($1/x$ weighted), intraday-apparent-recovery and -precision, interday-apparent-recovery and -precision, limit-of-quantification, and limit-of-detection evaluation for the LC-MS/MS validation of ZEN, α-ZEL, β-ZEL, ZEN-14-GlcA, α-ZEL-14-GlcA, β-ZEL-14-GlcA, ZEN14G, and ZEN14S in pig plasma; and linearity, recovery, standard errors of the mean, limits of detection, limits of quantification, signal suppression–enhancement, and total relative standard deviations of ZEN, α-ZEL, β-ZEL, α-ZAL, β-ZAL ZEN-14-GlcA, α-ZEL-14-GlcA, α-ZEL-7-GlcA, β-ZEL-14-GlcA, and β-ZEL-16-GlcA in pig plasma (PDF)

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Notes

■A. Callebaut died on June 3, 2017.

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■ ABBREVIATIONS USED

ZEN, zearalenone; α-ZEL, α-zearalenol; β-ZEL, β-zearalenol; ZEN14G, zearalenone-14-glucoside; ZEN14S, zearalenone-14-sulfate; GlcA, glucuronide; V_d, volume of distribution; CL, clearance; AUC, area under the curve; k_e, elimination rate constant; $T_{1/2el}$, elimination half-life; CONV, conversion; F, absolute oral bioavailability; FRAC, absorbed fraction; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LC-HRMS, liquid chromatography–high-resolution mass spectrometry; IS, internal standard

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