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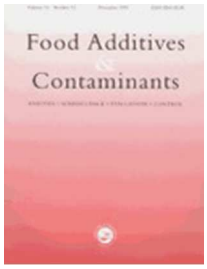

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Abstract:	In this study, three new models were developed for efficacy testing of mycotoxin detoxifying agents in relation to recent European guidelines. In the first model, deoxynivalenol was given to broiler chickens as an intra-crop bolus together with a mycotoxin detoxifying agent in order to study the plasma concentration-time profile of deoxynivalenol. In the second model the same oral bolus was given, preceded by an oral bolus of mycotoxin detoxifying agent, to make sure the detoxifying agent was present in the whole intestinal tract when the mycotoxin was administered.

	In the third model, the mycotoxin detoxifying agent was mixed in the feed of broiler chickens, and after one week feeding, deoxynivalenol was given as an oral bolus. In order to evaluate the efficacy of these agents, plasma concentration-time profiles were set up and the main toxicokinetic parameters were compared. Two commercially available mycotoxin detoxifying agents were tested, but they were not able to lower the oral availability of deoxynivalenol. As a positive control, activated carbon was used. We showed that activated carbon significantly reduces the absorption and oral availability of deoxynivalenol in all three models. Therefore, it can be concluded that these models are able to demonstrate the efficacy of mycotoxin detoxifying agents in relation to EFSA guidelines.

New bolus models for *in vivo* efficacy testing of mycotoxin detoxifying agents in relation to EFSA guidelines, assessed using deoxynivalenol in broiler chickens

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Abstract

In this study, three new models were developed for efficacy testing of mycotoxin detoxifying agents in relation to recent European guidelines. In the first model, deoxynivalenol was given to broiler chickens as an intra-crop bolus together with a mycotoxin detoxifying agent in order to study the plasma concentration-time profile of deoxynivalenol. In the second model the same oral bolus was given, preceded by an oral bolus of mycotoxin detoxifying agent, to make sure the detoxifying agent was present in the whole intestinal tract when the mycotoxin was administered. In the third model, the mycotoxin detoxifying agent was mixed in the feed of broiler chickens, and after one week feeding, deoxynivalenol was given as an oral bolus. In order to evaluate the efficacy of these agents, plasma concentration-time profiles were set up and the main toxicokinetic parameters were compared. Two commercially available mycotoxin detoxifying agents were tested, but they were not able to lower the oral availability of deoxynivalenol. As a positive control, activated carbon was used. We showed that activated carbon significantly reduces the absorption and oral availability of deoxynivalenol in all three models. Therefore, it can be concluded that these models are able to demonstrate the efficacy of mycotoxin detoxifying agents in relation to EFSA guidelines.

Keywords: mycotoxins; deoxynivalenol; efficacy testing; mycotoxin detoxifying agent; modeling; legal assessment

Introduction

The contamination of feed with mycotoxins is a continuing feed safety issue leading to economic losses in animal production (Wu, 2007). Consequently, a variety of methods for the decontamination of feed have been developed, but mycotoxin detoxifying agents seem to be the most promising and are therefore most commonly used (Jard, et al., 2011, Kolosova and Stroka, 2011) These detoxifying agents can be divided into two different classes, namely mycotoxin binders and mycotoxin modifiers. These two classes have different modes of action; mycotoxin binders adsorb the toxin in the gut, resulting in the excretion of complex toxin-binder in the faeces, whereas mycotoxin modifiers transform the toxin into non-toxic metabolites (EFSA, 2009). The extensive use of these additives has led, in 2009, to the establishment of a new group of feed additives: ‘substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action’ (European Commission, 2009).

Evidently, the efficacy of these products for their adsorbing or degrading ability should be tested. Many *in vitro* methods have been developed ranging from single-concentration studies to classical isotherm studies (binder concentration fixed, toxin concentration increasing) and beyond, to more complex set-ups such as gastro-intestinal tract models (EFSA, 2009). Nevertheless, in recent guidelines the European Food Safety Authority (EFSA) has stated that *in vitro* tests do not fully prove the efficacy of mycotoxin detoxifying agents (EFSA, 2009 and 2010) and that *in vivo* trials should be performed. Although, these *in vivo* trials can report non-specific parameters such as organ weight, performance parameters (e.g. growth rate, feed conversion rate) and blood serum parameters (e.g. total protein, albumin, key enzymes), they are not sufficient as proof of efficacy of mycotoxin detoxifying agents. In addition, specific parameters should be measured based on toxicokinetic studies including the bioavailability and absorption/excretion of the toxin. For each mycotoxin the EFSA has proposed specific end-points. For deoxynivalenol (DON), the most relevant end-point is measuring DON and its metabolites (deepoxy-deoxynivalenol or DOM-1, in particular) in blood plasma.

In their guidelines, the EFSA proposes short-term feeding trials in which the mycotoxin and detoxifying agent are mixed in the feed (steady-state design). In these experimental set-ups the pre-sampling period should not be shorter than seven days, and the blood samples should be collected over a five-day period during feeding (EFSA, 2010). However, these trials are labor intensive and quite complicated to perform. Moreover, in a three week feeding trial with broiler chickens where the maximum allowed level of 5 mg DON/kg feed (European Commission, 2006) was added to the feed, no plasma concentrations of DON and DOM-1 could be measured when sampled on a weekly basis (Osselaere, et al., 2012). This indicates that a model where DON and detoxifying agent are mixed in the feed, is not an appropriate way to prove the efficacy of mycotoxin detoxifying agents for DON in broiler chickens using EFSA end-points.

Therefore, we propose oral bolus models which are easy to perform, have a straightforward design and can easily be adopted by the feed additive producing industry. All three proposed models in our study are in relation to the EFSA guidelines, stating that specific parameters should be evaluated, based on *in vivo* toxicokinetic or ADME studies (absorption, distribution, metabolisation and excretion) (EFSA, 2010).

To the author's knowledge, no studies have been published yet according to these recent EFSA guidelines. Broiler chickens were chosen as they are convenient to handle and blood collections can easily be performed. Moreover, poultry meat represents one-third of all

meat produced globally, indicating the major importance of the broiler chicken industry (Scanes, 2007). As mycotoxin, deoxynivalenol was used as it is the most common mycotoxin found in European feed commodities. In a recent study it was found that 78% of European feed samples were contaminated with DON (Monbaliu, et al., 2010). Deoxynivalenol is produced by several fungi of the *Fusarium* genus and it impairs the protein synthesis by binding to the 60S ribosomal unit and therefore interferes with the activity of peptidyltransferase. Trichothecenes can also cause the 'ribotoxic stress syndrome' by activating mitogen-activated protein kinases (MAPKs) (Pestka, 2007). Poultry seem to be relatively resistant to DON compared to other species, especially pigs. Nevertheless, low to moderate levels of this toxin can cause several effects which influence immunological and performance parameters (Awad, et al., 2006).

In our models, we tested two commercially available mycotoxin detoxifying agents as to their ability to lower the oral bioavailability of DON. The first product was a mycotoxin binder, composed of esterified glucomannans derived from the cell wall of *Saccharomyces cerevisiae* yeasts. Unspecific *in vivo* trials have shown the ability of the glucomannan product to counteract the negative effects of DON on performance parameters and blood biochemical parameters in broiler chickens and pigs (Aravind, et al., 2003, Faixova, et al., 2006, Swamy, et al., 2004). The second product was a combination of mycotoxin binder and modifier. The bentonite fraction (binder) has a high affinity towards aflatoxins, but not towards DON (Avantaggiato, et al., 2005) as aflatoxins are hydrophilic planar structures with a high affinity for planar surfaces. In contrast, DON is a non-ionisable molecule with a more polar structure and a bulky epoxy group and therefore not easily bound by mycotoxin binders (EFSA, 2009). Nevertheless, this product also contains a yeast, claimed to be able to open the C-12,13 epoxide ring, converting DON into a non-toxic metabolite DOM-1 (Awad, et al., 2010, Diaz, et al., 2005). This mycotoxin detoxifying agent showed potential in diminishing the deleterious effects of DON on growth performance and other non-specific parameters in pigs (Plank, et al., 2009). However, Dänicke et al. (2003) could not show benefits of this detoxifying agent on performance and blood chemical parameters in poultry. As positive control, activated carbon was used as it proved to adsorb various compounds, including mycotoxins such as DON (Avantaggiato, et al., 2004, Cavret, et al., 2010).

Materials and methods

Animals and housing conditions

For each bolus model, thirty-two twenty-one-day-old healthy broiler chickens (Ross 308, Poeke, Belgium) were randomly allotted in 4 groups of eight chickens, males and females equally divided. The animals were housed in pens of 4 m^2/pen (8 animals/pen), one week before the start of the experiment to adapt to the environment. Blank feed was given *ad libitum* during the trial. The light schedule was 20 h light, 4 h darkness. The temperature was kept between 18 and 25°C. The relative humidity was between 40 and 80%. The bedding of the pens consisted of wood shavings, allowing the animals to perform their natural dust bathing and foraging behaviour. This experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine (Ghent University, number EC 2011-14).

Feed

Commercially available broiler feed (Bromix Plus®) was obtained from Versele-Laga (Deinze, Belgium). This feed was analyzed for the presence of mycotoxins by a validated multi-mycotoxin liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (Fytolab, Zwijnaarde, Belgium). The analyzed mycotoxins were aflatoxin B1, B2, G1 and G2, cytohalasin E, deoxynivalenol, 3-acetyl-deoxynivalenol, nivalenol, fumonisin B1 and B2, T-2 and HT-2 toxin, ochratoxin A, zearalenone, α- and β-zearalenol. The concentrations of the mycotoxins were all below the limit of detection (LOD), which was 100 µg/kg for DON, 3-acetyl-DON and nivalenol and between 0.5 and 50 µg/kg for the other mycotoxins. The animals received this blank feed during the complete trial.

Mycotoxins and detoxifying agents

Deoxynivalenol used for the animal experiments was purchased as a powder from Fermentek LTD (Jerusalem, Israel). The administered dose of 0.750 mg DON/kg BW was calculated based on the maximally allowed concentration in poultry feed, i.e. 5 mg/kg (European Commission, 2006), and the daily feed intake, i.e. 150 g/kg BW. The mycotoxin was dissolved in ethanol *pro analysi* and water of HPLC quality (1:8, v/v), in order to obtain a stock solution of 1 mg/mL, which was used for dosing the broiler chickens.

The standards of DON and DOM-1, used for the analytical experiments, were

purchased from Sigma-Aldrich (Bornem, Belgium) and were dissolved in acetonitrile (ACN) to obtain stock solutions of 1 mg/mL. Working solutions were used to prepare matrix-matched calibrators and quality control samples in plasma. These working solutions were prepared by mixing appropriate volumes of the stock solution with ACN and water (1:1, v/v), both of HPLC quality. The internal standard ($^{13}C_{15}$-DON, 25 µg/mL ACN) was obtained from Biopure (Tulln, Austria).

Two commercially available mycotoxin detoxifying agents were used. The first product was a mycotoxin binder, composed of esterified glucomannans derived from the cell wall of *Saccharomyces cerevisiae* yeasts. The second product was a combination of a mycotoxin binder (i.e. bentonite) and a modifier (a yeast). The mycotoxin detoxifying agents were administered at a dose of 1 g/kg BW for the intra-crop bolus. The negative control group was given blank feed (1 g/kg BW) instead of a detoxifying agent. Both detoxifying agent and blank feed were suspended in 5 mL of water in a syringe immediately before administration into the crop, and flushed afterwards with 1 mL of water. This administration was performed using the tubing of a catheter (14G, 2", Vasofix® Braunüle®) (Braun, Melsungen, Germany). The positive control group received activated carbon (AC) (1 g/kg BW) (NORIT Carbomix®, KELA Pharma, Sint-Niklaas, Belgium) suspended in water, also by means of an intra-crop bolus.

Study design

Bolus model 1

The animals were divided into four groups of eight animals. Each group received a different treatment. The animals in the Detoxifying Agent 1, Detoxifying Agent 2, Negative Control and Positive Control group received a bolus of DON and mycotoxin detoxifying agent 1, DON and mycotoxin detoxifying agent 2, DON and blank feed and DON and activated carbon, respectively. Feed was withheld for 12 h before the bolus administration, until 4 h post-administration.

Following the administration, blood samples were taken from the leg vein at different time points, at 0 (just before bolus administration), 15, 30, 45 min, 1, 1.5, 2, 2.5, 3, 4, 6 and 8 h post-administration. The samples were centrifugated (3500 rpm, 10 min, 4°C), and plasma was stored at ≤ -15°C until further analysis.

Bolus model 2

The experiment was similar to experiment 1, except that the mycotoxin detoxifying agent was now not only given at the same time of the DON bolus, but also 1 and 2 hours before the DON administration as an intra-crop bolus ('preload' of the animals with the detoxifying agent).

Bolus model 3

In this experiment the mycotoxin detoxifying agents were mixed in the blank feed at a dose of 2 kg/ton feed, as recommended by the manufacturers. This feed was given from the start of the experiment onwards, i.e. one week before the bolus administration, until the last blood sampling point (8 h post-bolus administration). In this experiment there was no special feed deprivation period.

Plasma analysis

The plasma concentrations of DON and DOM-1 were determined by LC-MS/MS, based on a validated method with modifications in the sample preparation and chromatography set-up (De Baere, et al., 2011). Briefly, 250 µl of plasma was spiked with 12.5 µl working solution (1 µg/mL) of internal standard (IS) ($^{13}C_{15}$-DON). This was followed by adding 750 µl of ACN. Next, the samples were vortexed (15 sec) and centrifugated (10 min, 13000 rpm, 4°C). The supernatant was then evaporated using a gentle nitrogen stream (40 ± 5°C). The dry residue was reconstituted in 200 µl of a 95/5 (v/v) mixture of mobile phase A/B. The mobile phase A consisted of 0.1 % glacial acetic acid in water of UPLC quality. Mobile phase B consisted of methanol of UPLC quality. After vortex mixing and filtering through a Millex® filter (0.22 µm), the sample was transferred to an autosampler vial, and an aliquot (10 µl) was injected onto the LC-MS/MS instrument.

The LC system consisted of a quaternary, low-pressure mixing pump with vacuum degassing, type Surveyor MSpump Plus and an autosampler, type Autosampler Plus, from ThermoFisher Scientific (Breda, The Netherlands). Chromatographic separation was achieved on a Hypersil-Gold column (50 mm x 2.1 mm i.d., dp: 1.9 µm) in combination with a guard column of the same type, both from Interscience (Louvain-la-Neuve, Belgium). A gradient elution was performed: 0-1 min (95% A/5% B), 4 min (linear gradient to 80% B), 4-5.1 min (20% A/80% B), 5.6 min (linear gradient to 95% A), 5.6-8 min (95% A/5% B). The flow rate

was 300 µl/min. The LC column effluent was interfaced to a TSQ® Quantum Ultra triple quadrupole mass spectrometer, equipped with a heated electrospray ionization (h-ESI) probe (ThermoFisher Scientific), operating in the negative ionization mode. Following selected reaction monitoring (SRM) transitions were monitored and used for quantification: for DON m/z 355.1 > 265.2 and 355.1 > 295.1, for DOM-1 m/z 339.1 > 59.1 and 339.1 > 249.0 and for $^{13}C_{15}$-DON m/z 370.1 > 279.2 and 370.1 > 310.1. The limit of quantification (LOQ) of DON and DOM-1 was 1 ng/mL and the limit of detection (LOD) of DON 0.1 ng/mL and 0.19 ng/mL of DOM-1.

Toxicokinetic and statistical analysis

The following toxicokinetic parameters were calculated (WinNonlin 6.2.0, Phoenix, Pharsight corp., USA) using non-compartmental analysis: area under the plasma concentration-time curve from time 0 to infinite ($AUC_{0\text{-}inf}$), maximal plasma concentration (C_{max}), time to maximal plasma concentration (T_{max}), elimination half-life ($T_{1/2el}$), elimination rate constant (k_{el}) and relative oral bioavailability (relative OBB). This relative OBB was calculated according to the formula: relative $OBB = \frac{AUC0-inf\ (DON+Detoxifying\ Agent)}{AUC0-inf\ (DON)}$ x 100. Using non-compartmental analysis, a better estimate of C_{max} could be made, which is of great importance for the interpretation of the data. The absorption rate constant (k_a) and the absorption half-life ($T_{1/2a}$) were calculated using one-compartmental analysis. The statistical analysis was performed with SPSS via one-way ANOVA (SPSS 17.0, IBM, USA). The significance level was set at 0.05.

Results

The plasma concentration-time profiles of DON after bolus administration with or without detoxifying agent (model 1), after bolus administration preceded by preload with blank feed or a detoxifying agent (model 2) and after a bolus administration of DON preceded by one week feeding of blank feed with or without mycotoxin detoxifying agent added, are shown in Figure 1. The main toxicokinetic parameters are summarized in Table 1. Plasma concentrations of the main metabolite of DON, DOM-1, were not detected. Moreover, the concentration of DON in all samples of the positive control group (DON+AC) were below LOQ and therefore, no toxicokinetic parameters could be calculated for this group.

Table 1. Main toxicokinetic parameters of DON after administration of DON and blank feed (negative control) or DON and a detoxifying agent (detoxifying agent 1 or 2) in broiler chickens (n=8), using model 1, 2 and 3. Results are given as mean values ± SD.

Figure 1. Plasma concentration-time profile of DON after administration of DON and blank feed (negative control) or DON and a detoxifying agent (detoxifying agent 1 or 2) in broiler chickens (n=8), using model 1, 2 and 3. Results are presented as mean values + SD.

Discussion

Up till now, no straightforward models for *in vivo* efficacy testing of mycotoxin detoxifying agents, in relation to the recent EFSA guidelines, have been reported. No studies have been published on the ability of mycotoxin detoxifying agents to lower the oral bioavailability of mycotoxins in poultry. Dänicke et al. (2001) studied the excretion kinetics of zearalenone (ZON) in broiler chickens and the efficacy of a mycotoxin detoxifying agent to alter the excretion of ZON. No difference in toxicokinetic parameters were found after bolus administration of ZON with or without the mycotoxin detoxifying agent. The enterohepatic recirculation of ZON and the rapid passage of the detoxifying agent through the intestinal tract was put forward as a possible explanation.

Previous studies (Döll, et al., 2004, Sabater-Vilar, et al., 2007) have evaluated the *in vitro* binding or biotransforming ability of different mycotoxin detoxifying agents, including those used in this study. In those screening studies, none of the tested products were able to effectively bind DON, except for activated carbon. These *in vitro* findings correlate with our findings, where no significant differences in toxicokinetic parameters were found between the detoxifying agent groups and the negative control group, except in the first bolus model. Surprisingly, a significant higher $AUC_{0\text{-}inf}$, C_{max} and k_a, a shorter $T_{1/2a}$ and lower relative OBB were found in the detoxifying group 1 compared with the negative control group. However, this relates with the study by Goossens et al. (2012) in which the interaction between a yeast derived mycotoxin detoxifying agent and the antibiotic doxycycline was investigated in pigs. It was found that the detoxifying agent, in combination with T-2 toxin, enhanced the oral absorption of the drug. A recent study showed a significant influence of a mycotoxin detoxifying agent on the oral absorption of oxytetracycline in broiler chickens (Osselaere, et al., 2012). Again, an increased oral bioavailability in the detoxifying agent group was seen. The mechanisms of this interaction still have to be elucidated and are currently being investigated. Most probably, these effects are not related to a direct interaction between drug

and detoxifying agent. Possible indirect effects such as promotion of intestinal health, altered intestinal immunological parameters, influence on intestinal mucus production, etc. can be put forward.

In the present study, activated carbon was used as a positive control. This product is a basic universal antidote which adsorbs various compounds, including mycotoxins such as DON (Avantaggiato, et al., 2004, Cavret, et al., 2010). However, the commercial use of AC in practice should be avoided in order to minimize the risk of a diminished nutrient absorption as well as the impairment of nutritional value (Avantaggiato, et al., 2004, Ramos, et al., 1996). In all of the three bolus models, the plasma concentration of DON was below LOQ, indicating the efficient adsorption of DON by AC in the intestinal tract. Therefore, we can conclude that the 3 models developed in this study are able to demonstrate the (in)efficacy of mycotoxin detoxifying agents. Further research should be performed on testing these models with other mycotoxins and detoxifying agents.

Conclusions

It can be stated that three suitable *in vivo* models for efficacy testing of mycotoxin detoxifying agents were developed. The reliability of the models was demonstrated using activated carbon. The two mycotoxin detoxifying agents used in this study were not able to lower the oral bioavailability of DON.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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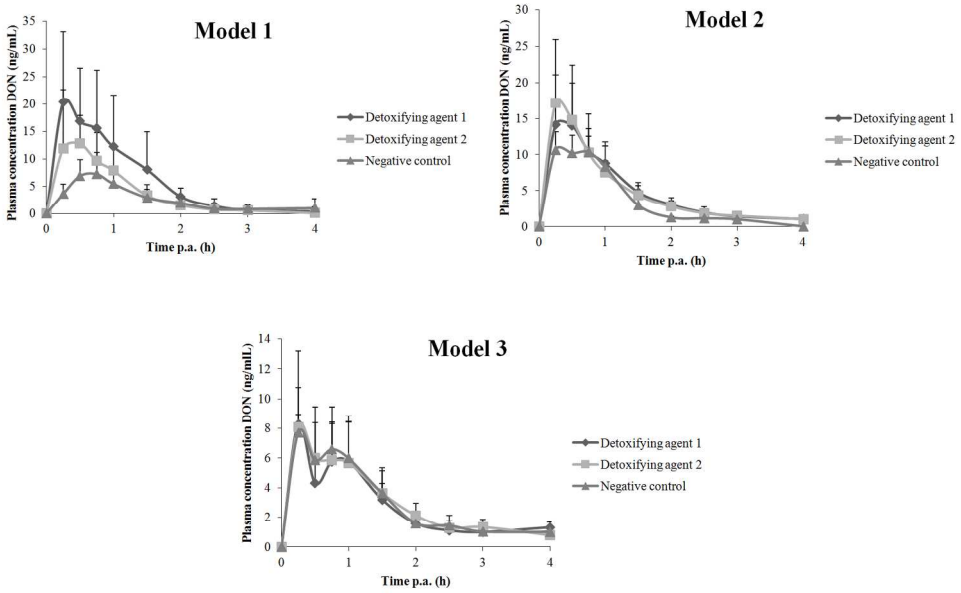
Figure caption

Figure 1. Plasma concentration-time profile of DON after administration of DON and blank feed (negative control) or DON and a detoxifying agent (detoxifying agent 1 or 2) in broiler chickens (n=8), using model 1, 2 and 3. Results are presented as mean values + SD.

Table caption

Table 1. Main toxicokinetic parameters of DON after administration of DON and blank feed (negative control) or DON and a detoxifying agent (detoxifying agent 1 or 2) in broiler chickens (n=8), using model 1, 2 and 3. Results are given as mean values ± SD.



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	Model 1			Model 2			Model 3		
Toxicokinetic parameter	Negative control	Detoxifying agent 1	Detoxifying agent 2	Negative control	Detoxifying agent 1	Detoxifying agent 2	Negative control	Detoxifying agent 1	Detoxifying agent 2
$AUC_{0\text{-}inf}$ (ug.h/mL)	10.22 ± 3.15	24.71 ± 10.75*	15.60 ± 5.91	14.86 ± 2.66	20.23 ± 5.20	19.42 ± 3.50	12.13 ± 4.99	10.58 ± 1.68	12.02 ± 5.77
C_{max} (ng/mL)	8.22 ± 2.69	23.74 ± 12.00*	15.21 ± 6.11	14.13 ± 2.25	19.26 ± 7.25	22.56 ± 10.43	12.22 ± 7.42	9.83 ± 4.06	9.68 ± 6.28
T_{max} (h)	0.66 ± 0.16	0.59 ± 0.28	0.47 ± 0.22	0.50 ± 0.25	0.50 ± 0.19	0.38 ± 0.16	0.57 ± 0.22	0.53 ± 0.35	0.69 ± 0.27
k_a (h^{-1})	1.76 ± 0.29	10.48 ± 10.40*	3.90 ± 2.27*	17.20 ± 19.50	11.14 ± 10.76	24.66 ± 22.37	13.19 ± 5.98	8.72 ± 4.05	10.04 ± 3.52
$T_{1/2a}$ (h)	0.41 ± 0.07	0.22 ± 0.18*	0.26 ± 0.13*	0.20 ± 0.16	0.22 ± 0.15	0.18 ± 0.16	0.29 ± 0.19	0.27 ± 0.21	0.32 ± 0.15
k_{el} (h^{-1})	1.29 ± 0.48	1.70 ± 0.45	1.47 ± 0.45	0.95 ± 0.48	1.04 ± 0.30	0.95 ± 0.13	1.24 ± 0.25	1.02 ± 0.28	1.01 ± 0.29
$T_{1/2el}$ (h)	0.70 ± 0.27	0.44 ± 0.11	0.57 ± 0.21	0.73 ± 0.37	0.80 ± 0.29	0.75 ± 0.11	0.59 ± 0.13	0.82 ± 0.31	0.86 ± 0.38
Relative OBB (%)		256 ± 109*	153 ± 58		136 ± 35	131 ± 24		87 ± 14	99 ± 47

$AUC_{0\text{-}inf}$ = area under the plasma concentration-time curve from time 0 to infinite; C_{max} = maximal plasma concentration; T_{max} = time to maximal plasma concentration; k_a = absorption rate constant; $T_{1/2a}$ = absorption half-life; k_{el} = elimination rate constant; $T_{1/2el}$ = elimination half-life; OBB = oral bioavailability; the asterix (*) indicates a significant difference ($p<0.05$) compared to the negative control group

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