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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.

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	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.

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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, metabolism 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

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

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

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115 **Materials and methods**

116 ***Animals and housing conditions***

117 For each bolus model, thirty-two twenty-one-day-old healthy broiler chickens (Ross 308,
118 Poeke, Belgium) were randomly allotted in 4 groups of eight chickens, males and females
119 equally divided. The animals were housed in pens of 4 m²/pen (8 animals/pen), one week
120 before the start of the experiment to adapt to the environment. Blank feed was given *ad*
121 *libitum* during the trial. The light schedule was 20 h light, 4 h darkness. The temperature was
122 kept between 18 and 25°C. The relative humidity was between 40 and 80%. The bedding of
123 the pens consisted of wood shavings, allowing the animals to perform their natural dust
124 bathing and foraging behaviour. This experiment was approved by the Ethical Committee of
125 the Faculty of Veterinary Medicine (Ghent University, number EC 2011-14).

126 ***Feed***

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

136 ***Mycotoxins and detoxifying agents***

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

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

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144 purchased from Sigma-Aldrich (Bornem, Belgium) and were dissolved in acetonitrile (ACN)
145 to obtain stock solutions of 1 mg/mL. Working solutions were used to prepare matrix-
146 matched calibrators and quality control samples in plasma. These working solutions were
147 prepared by mixing appropriate volumes of the stock solution with ACN and water (1:1, v/v),
148 both of HPLC quality. The internal standard (¹³C₁₅-DON, 25 µg/mL ACN) was obtained from
149 Biopure (Tulln, Austria).

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

162 **Study design**

163 *Bolus model 1*

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

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

174 *Bolus model 2*

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

179 *Bolus model 3*

180 In this experiment the mycotoxin detoxifying agents were mixed in the blank feed at a dose of
181 2 kg/ton feed, as recommended by the manufacturers. This feed was given from the start of
182 the experiment onwards, i.e. one week before the bolus administration, until the last blood
183 sampling point (8 h post-bolus administration). In this experiment there was no special feed
184 deprivation period.

185 *Plasma analysis*

186 The plasma concentrations of DON and DOM-1 were determined by LC-MS/MS, based on a
187 validated method with modifications in the sample preparation and chromatography set-up
188 (De Baere, et al., 2011). Briefly, 250 µl of plasma was spiked with 12.5 µl working solution
189 (1 µg/mL) of internal standard (IS) ($^{13}\text{C}_{15}$ -DON). This was followed by adding 750 µl of
190 ACN. Next, the samples were vortexed (15 sec) and centrifugated (10 min, 13000 rpm, 4°C).
191 The supernatant was then evaporated using a gentle nitrogen stream ($40 \pm 5^\circ\text{C}$). The dry
192 residue was reconstituted in 200 µl of a 95/5 (v/v) mixture of mobile phase A/B. The mobile
193 phase A consisted of 0.1 % glacial acetic acid in water of UPLC quality. Mobile phase B
194 consisted of methanol of UPLC quality. After vortex mixing and filtering through a Millex®
195 filter (0.22 µm), the sample was transferred to an autosampler vial, and an aliquot (10 µl) was
196 injected onto the LC-MS/MS instrument.

197 The LC system consisted of a quaternary, low-pressure mixing pump with vacuum
198 degassing, type Surveyor MSpump Plus and an autosampler, type Autosampler Plus, from
199 ThermoFisher Scientific (Breda, The Netherlands). Chromatographic separation was achieved
200 on a Hypersil-Gold column (50 mm x 2.1 mm i.d., dp: 1.9 µm) in combination with a guard
201 column of the same type, both from Interscience (Louvain-la-Neuve, Belgium). A gradient
202 elution was performed: 0-1 min (95% A/5% B), 4 min (linear gradient to 80% B), 4-5.1 min
203 (20% A/80% B), 5.6 min (linear gradient to 95% A), 5.6-8 min (95% A/5% B). The flow rate

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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 ¹³C₁₅-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-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:
$$\text{relative OBB} = \frac{\text{AUC}_{0-\text{inf}}(\text{DON} + \text{Detoxifying Agent})}{\text{AUC}_{0-\text{inf}}(\text{DON})} \times 100$$
. 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 $\pm$ 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 $\pm$ 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-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

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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.

518x328mm (96 x 96 DPI)

Toxicokinetic parameter	Model 1			Model 2			Model 3		
	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-∞} (ng.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 _{1/2α} (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 _d (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 _{1/2β} (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-∞} = 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_{1/2α} = absorption half-life; k_d = elimination rate constant; T_{1/2β} = elimination half-life; OBB = oral bioavailability; the asterix (*) indicates a significant difference (p<0.05) compared to the negative control group

371x146mm (96 x 96 DPI)