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Pharmacokinetics of gallium nitrate after oral administration in adult horses – pilot study

G. F. POLLINA*
G. ZAGOTTO†
P. MARITAN†
I. IACOPETTI‡ &
R. BUSETTO‡

**DVM, Practitioner, Italy; †Department of Pharmaceutical Sciences, University of Padua, Italy; ‡Department of Veterinary Clinical Sciences, University of Padua, Italy*

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Gallium (Ga), a metal in group IIIA of the periodic table, has shown a remarkable activity against bone resorption and could therefore possibly prove useful in the treatment of certain diseases in sport horses, for example navicular disease. The aim of this study was to gain more information concerning the kinetics of Ga after oral administration of gallium nitrate (GaN) in adult horses. Six horses received a single dose of 10 mg/kg of GaN mixed with the food ration. Absorption was slow (T_{max} = 10 ± 3 h, $T_{½abs}$ = 2 ± 0.8 h), and a C_{max} of 26 ± 11 μg/L was achieved. Excretion followed a one-phase elimination model, with a long half-life ($T_{½el}$ = 52 ± 14 h). By means of a mathematical model, we estimated that the plasmatic levels should reach 93 μg/L (1.33 μM) at steady state, following the repeated daily administration of 10 mg/kg of GaN. A three times lower concentration has been demonstrated as effective in inhibiting the osteolytic activity of osteoclasts *in vitro*. The results of this study suggest that the administration of oral GaN at a rate of 10 mg/kg per day may be considered for future clinical studies.

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Dr. Gilberto F. Pollina, Via Ca' Savorgnan 23, 30172, Venezia, Italy. E-mail: bertogil@inwind.it

INTRODUCTION

Navicular disease is a major cause of forelimb lameness in the horse and remains one of the most frustrating diseases for owners, trainers and veterinarians. It is estimated that this disease is responsible for about one-third of all chronic forelimb lameness in horses (Stashak, 2002). It is a progressive degenerative disease of the podotrochlear apparatus, an anatomic complex located in the palmar aspect of the foot. The podotrochlear apparatus consists of the navicular bone (NB), the paired collateral sesamoidean ligaments (CSLs) of the NB, the distal sesamoidean impar ligament (DSIL) of the NB, the podotrochlear bursa (PB) and the deep digital flexor tendon (DDFT). The dorsal surface and the distal border of the NB articulate with the middle phalanx and the distal phalanx, respectively, forming the distal interphalangeal joint. The aetiopathogenesis of navicular disease is not completely understood. However, the most likely hypotheses include genetic predisposition (Diesterbeck & Distl, 2007), navicular bone shape predisposition (Dik *et al.*, 2001), biomechanical alterations (McGuigan & Wilson, 2001; Wilson *et al.*, 2001) caused by poor foot conformation, incorrect shoeing and/or excess of work (Trotter, 2001). In the presence of navicular disease, the structures composing the podotrochlear apparatus, especially the DDFT and the NB, undergo degenerative and inflammatory processes (Dyson *et al.*, 2005). One of the characteristics that stands out in the histopathological picture and magnetic resonance imaging and that is frequently related to the presence of pain is the osteolytic degeneration of the NB (Murray *et al.*, 2006; Jenner & Kirker-Head, 2011), in which the metabolic balance between bone anabolism and catabolism is affected. To date, it has not yet been entirely clarified whether NB lesions are primary or secondary to the lesions involving the adjacent structures (DDFT, CSLs, DSIL and PB). Navicular disease is particularly insidious, because of sluggish onset and difficult and expensive diagnosis. The treatments currently available are mostly palliative and are a cause of temporary (medical treatment) or permanent (surgical treatment) exclusion from competition (source: FEI, 2010).

In 2003, based on the results of a clinical study conducted by a French/Italian team of researchers (Denoix *et al.*, 2003), a new class of drugs was introduced in the therapeutic arsenal for navicular disease: bisphosphonates. Bisphosphonates are molecules responsible for antiresorptive activity. Already in clinical use as bone resorption inhibitors, their use in navicular disease is aimed at reducing the severity of bone lesions and improving the clinical picture. However, the clinical benefits of bisphosphonate administration in horses with navicular disease were evident

only in a small proportion of the animals treated and only in horses in which lameness had arisen <6 months prior to treatment (Kamm *et al.*, 2008).

Another active substance with potent antiresorptive activity is gallium (Ga), a post-transition metal belonging to group IIIA of the periodic table. In terms of chemical properties, it has strong similarities with ferric iron (Fe^{3+}), aluminium (Al), zinc (Zn) and indium (In). The chemical behaviour of Ga is close to that of Fe^{3+} in terms of electric charge, ionic radius, coordination number and electronic configuration (Collery *et al.*, 2002). Unlike iron, Ga is unable to attain a divalent state under physiological conditions, preventing it from participating in redox reactions and from entering those Fe^{2+}-binding molecules such as heme (Logan *et al.*, 1981 in Bernstein, 1998). Similarly to iron, Ga has a strong affinity for transferrin (Vallabhajosula *et al.*, 1980; Jackson & Byrne, 1996; Rudnev *et al.*, 2006; Chikh *et al.*, 2007) and binds to it slowly (10–40 min is required to reach the equilibrium *in vitro*) but tightly (Harris & Pecoraro, 1983). In humans, it has been estimated that when at equilibrium, with total Ga plasma concentrations standing at just under 20 μM, 99.9% of Ga is bound to transferrin (Bernstein, 1998). This factor plays an important role in understanding the kinetics of Ga in mammals.

Gallium, like bisphosphonates, is employed in human medicine to normalise bone metabolism, through down-regulation of osteoclast degranulation. Many studies have been conducted on the efficacy of Ga in inhibiting osteoclast activity, both *in vivo* and *in vitro*. Some of these are mentioned here below. Hall and Chambers (1990) found that osteoclast resorption activity is markedly inhibited *in vitro* by Ga concentrations of 0.39 μM and above in culture medium. Bockman (1991) demonstrated that Ga, at a concentration of 1 μg/mL (about 14 μM) *in vitro*, is capable of significantly inhibiting bone resorption induced by parathormone or tumour necrosis factor. Blair *et al.* (1992) noticed that bone slices in culture with viable osteoclasts, when pre-incubated with increasing concentrations of Ga, showed a dose-dependent inhibition of osteolysis starting at concentrations as low as 1 μM of Ga in the incubation medium. Verron *et al.* (2010) carried out an in-depth study on the working mechanism of Ga in inhibiting osteoclast-mediated bone resorption. This research suggested Ga has a regulatory effect both on mature osteoclasts, by inhibiting enzymatic synthesis and degranulation, as already reported in previous studies (Bockman, 1991, 2003), and on their precursors in the monocytic line, by inhibiting differentiation into osteoclasts, through a regulatory effect on gene transcription. The bone resorption inhibition by mature osteoclasts was considered significant with a Ga concentration of 10 μM and above in the medium and the inhibition of differentiation of their precursors was considered significant at concentrations of 2.5 μM and above in culture medium. Verron's study aimed to explore the potential applications of Ga in the treatment of osteoporosis in man: in their conclusions, the authors indicate this metal as a potential alternative to current treatments. In clinical trials, Warrell *et al.* (1986) found that a plasma Ga concentration of 0.92 μg/mL (about 13 μM) and above is sufficient to achieve acute normalisation of calcemia in human patients affected by paraneoplastic hypercalcemia. Bockman *et al.* (1995) performed a multi-centre trial on the use of GaN in human patients with advanced Paget's disease. Subcutaneous administration of 0.25 mg/kg per day of GaN (equivalent to 0.068 mg/kg per day of elemental Ga) was able to significantly reduce the levels of two bone resorption markers in adults: the serum level of alkaline phosphatase and the creatinine-normalised urinary level of bone collagen cross-linked peptide. The authors did not report the plasmatic levels of Ga in that study. However, they state in the discussion that the plasma concentrations achieved during the experiment were lower than 1/200 of 300 μM, which is in the order of 1 μM.

In a similar manner as proposed for bisphosphonates (Denoix *et al.*, 2003), we considered the use of Ga for the treatment of navicular disease. A trial conducted in the United States on horses with navicular disease reported encouraging results following repeated oral administration of 10 mg/kg per day of gallium nitrate (GaN) for a period of 4 months (Eby, 2006, unpublished data). The purpose of the present study is to acquire data on the kinetics of Ga, orally administered to adult horses in the form of GaN.

MATERIALS AND METHODS

The gallium nitrate for the research was supplied by Eby Pharma LLC (Austin, TX, USA) and was accompanied by a 4 N (>99.99%) purity certificate provided by Recapture Metals Inc, Blanding, UT, USA. The gallium was supplied as a 42% (w/v) solution of GaN in 500-mL sealed bottles. Before starting the experiment, we proceeded to titrate the solution by atomic absorption spectrometry and we found the concentration to be 42.1 ± 0.4% (10 determinations).

Horse selection

Six horses of adult age were selected for this study. Only horses considered free from disease, or other abnormalities potentially affecting the absorption, distribution, metabolism and excretion parameters regarding orally administered drugs were accepted. The inclusion assessment involved physical examination of the digestive system, cardio-circulatory system and excretory apparatus. Blood samples were also taken for haemato-biochemical screening including the following parameters: WBC, RBC, Hb, Hct, MCV, MCH, MCHC, Plt, Neu, Lym, Mon, Eos, Bas, fibrinogen, GOT, GPT, GGT, ALP, bilirubin, CK, urea, Cre, Glc, Ca, P, Mg, Na, Cl and K. None of the horses were given medication or food supplements from 48 h prior to GaN administration until the end of the test. An alphanumeric code (PK1, PK2, PK3, PK4, PK5, PK6) was assigned to each horse in the experiment.

Gallium nitrate administration

At least 24 h prior to drug administration, the horses were admitted to the facilities at the Department of Veterinary Clinical

Sciences, University of Padua, where the experiment took place. During this period, clinical examination was carried out and blood count and biochemical tests were performed. The dose administered to each subject was 10 mg/kg of GaN (equivalent to 2.73 mg/kg of elemental Ga). The horses were weighed using scales. Before the GaN administration, the horses were fasted from solid food for a period of 12 h and only 15 min prior to the gallium bolus was a ration of hay given, to stimulate peristalsis. GaN administration occurred in the form of 1% aqueous solution; the product was mixed with a quantity of crushed barley equal to 2‰ of the live weight of the animal and placed in a plastic manger. It was checked that the animals consumed the entire ration. All the horses consumed the whole ration, without interruption, taking between 5 and 10 min. The horses were not fed solid foods during the 6 h following drug administration. The horses could drink freely throughout the experiment.

Blood sample collection and storage

On the basis of existing literature on the kinetics of Ga administered orally in other species (e.g. Collery *et al.*, 1989; Bernstein *et al.*, 2000; Collery *et al.*, 2002; Martens *et al.*, 2007) and based on our preliminary results, we decided to take blood plasma samples for Ga quantification at the following times: 0′, 15′, 30′, 1, 2, 4, 8, 12, 16, 20, 24, 28, 32, 48, 72 and 96 h. Collection was carried out by means of a venous catheter placed in the jugular vein, and blood was transferred into a test tube containing EDTA as anticoagulant. Immediately after collection, blood was centrifuged at 3700 ***g*** for 6 min and the plasma was separated and poured into polypropylene tubes, which were immediately stored at −80 °C. Plasma analysis was performed within 4 weeks of collection.

Plasma sample analysis

A graphite furnace atomic absorption spectrometer (GFAAS) was used to quantify the Ga in the plasma. Using this technique, the detection limit and the quantification limit for nondiluted plasma were 1.5 and 3 μg/L, respectively. The plasma concentration was established on the basis of three measurements for each sample. The standard error was always lower than 5% for samples with concentrations above the quantification limit. Sample analysis was carried out using Varian AA240Z® GFAAS equipment (Agilent Technologies, Santa Clara, CA, USA).

Pharmacokinetic data analysis

The data obtained were processed with a nonlinear fitting software Sigmaplot® 9.0 (Systat Software Inc., Chicago, IL, USA), to extract the values of C_{max}, T_{max}, $T_{½abs}$, $T_{½el}$ and $AUC_{0-\infty}$.

RESULTS

The plasma Ga concentrations over time for each horse are illustrated in Fig. 1a. Table 1 shows the average plasma Ga concentration at the times indicated and their standard deviations. The data obtained were processed with a nonlinear fitting software (Sigmaplot® 9.0) to obtain the values of T_{max}, C_{max}, $T_{½abs}$, $T_{½el}$ and $AUC_{0-\infty}$. To adjust the concentrations over time, we selected a three-parameter bi-exponential formula:

Table 1. Mean plasma Ga concentrations at times indicated in adult horses following a single oral administration of 10 mg/kg of gallium nitrate, with standard deviations and relative standard deviations (n = 6)

Time (h)	Plasma Ga (μg/L)	SD (μg/L)	RSD (%)
0	0	0	0.00
0.5	2.69	2.31	85.90
1	7.41	3.81	51.43
2	15.09	7.62	50.49
4	21.41	9.49	44.33
8	25.36	9.84	38.81
12	25.27	11.27	44.60
16	24.18	10.30	42.58
20	23.33	10.20	43.71
24	23.15	9.17	39.59
28	21.74	10.16	46.75
32	20.43	9.98	48.88
48	15.23	6.90	45.30
72	11.65	5.14	44.08
96	9.15	3.83	41.84

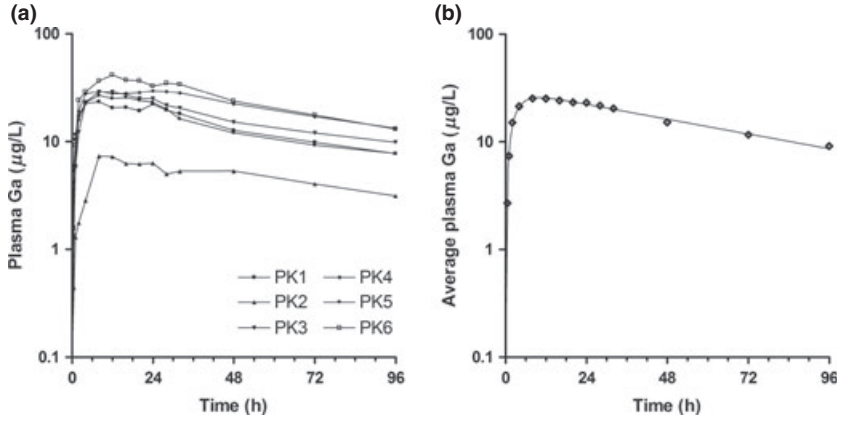

Fig. 1. Graphical representation of oral GaN kinetics. (a) Plasma Ga concentration vs. time curves for six adult horses following a single oral administration of 10 mg/kg of gallium nitrate. (b) Plasma Ga concentration vs. time curve, fitted on the average concentrations for six horses (data reported in Table 1). To fit the data, the following three-parameter bi-exponential equation has been used: $c = a * (e^{-\beta t} - e^{-\alpha t})$, in which a = 30.71, α = 0.331 and β = 0.0132. r^2 = 0.992.

$c = a * (e^{-\beta t} - e^{-\alpha t})$, because this was the most appropriate model for summarising the data trend (fitted curve and calculated parameters are presented in Fig. 1b). The Ga concentration curves in five of six horses showed a good degree of correlation with the equation chosen ($r^2 > 0.95$). Only in one horse (PK2) was the correlation degree lower ($r^2 = 0.917$). The correlation coefficient of the curve constructed on the average concentrations was equal to 0.992. In conclusion, oral administration of 10 mg/kg of GaN showed a mean C_{max} of 25.79 ± 11 μg/L, a T_{max} of 10.13 ± 3 h, a $T_{½abs}$ of 2.09 ± 0.78 h, a $T_{½el}$ of 52.43 ± 14 h and $AUC_{0-\infty}$ = 2230 ± 1073 μg/L·h.

It was not possible in this study to proceed with the repeated administration of GaN. However, by means of a mathematical model based on the superposition principle, we estimated the expected plasma Ga concentrations after repeated daily oral administration of GaN. As can be seen from the graph (Fig. 2), once a steady state is reached, Ga concentrations would stand at between a $C_{max(ss)}$ of 100.13 μg/L and a $C_{min(ss)}$ of 82.2 μg/L, with a $C_{ave(ss)}$ of 92.9 μg/L (1.33 μM). It is calculated that 99% of the steady-state concentration would be reached in 15 days.

DISCUSSION

This work stands as the first investigation into the kinetics of Ga administered orally to adult horses in the form of nitrate salt. The detected kinetics can be represented almost perfectly ($r^2 = 0.992$) by a three-parameter bi-exponential equation $c = a * (e^{-\beta t} - e^{-\alpha t})$. There is no evidence of a clear distribution/rapid elimination phase, which is presumed as integrated within the stages of absorption and early elimination. The elimination half-life was very long (52.4 h), concurring with the findings in other mammals, where it ranged between 20 and 100 h, depending on the animal species considered and administration route (Collery *et al.*, 1989; Bernstein *et al.*, 2000; Martens *et al.*, 2007). During the first 48–72 h following oral administration of GaN, the half-life of Ga elimination is longer than that following intravenous GaN administration (Bernstein *et al.*, 2000). This phenomenon may be explained by the observation that, despite the very high affinity with transferrin, Ga is likely to bind to it very slowly (Harris & Pecoraro, 1983; see Introduction); hence, parenteral administration leads, at least in the first instance, to a massive formation of gallates, $Ga(OH)_4^-$, which are rapidly excreted by the kidneys.

On the other hand, the slow absorption of oral GaN leads to Ga entering the bloodstream more gradually, thus enabling a larger portion of it to bind to the TF, which is retained by the glomerular filter. Bruner *et al.* (1953a,b) showed that Ga excretion following intravenous administration occurs either via the kidneys or via the enteric tract: during the first 24 h following administration (when most of the Ga in the blood exists in gallate form), elimination occurs almost entirely through the kidneys, whereas the enteric elimination route becomes increasingly important in the following days (as most of plasma Ga exists in the form of TF-bound Ga).

Therefore, it seems logical to conclude that the slower Ga enters the bloodstream, the greater the proportion excreted by the enteric tract and the slower the elimination.

A small peak in concentration deviating slightly from the expected curve was noted in five of six horses between 24 and 28 h following administration. This slight deviation can probably be explained by the presence of an enterohepatic recirculation or by the presence of various intestinal sites with differing Ga absorption capacities.

The Ga concentrations measured after oral administration are very low if compared to the total dose administered, suggesting a poor bioavailability of GaN. Because the distribution volume (V_d) in the equine species is not known, as intravenous studies in the horse do not exist, it is not possible here to determine GaN bioavailability. However, based on data for dog and man, and thus considering a V_{ss} (distribution volume at steady state) of between 13 and 38 times the plasma volume (Krakoff *et al.*, 1979; Kelsen *et al.*, 1980; Bernstein *et al.*, 2000), we can reasonably expect a bioavailability ranging between 0.7% and 2%.

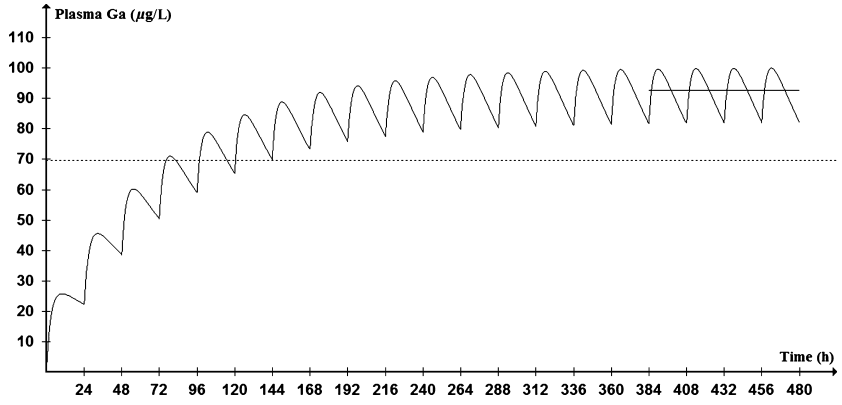

Fig. 2. Estimated gallium concentrations in plasma, following repeated oral administration of 10 mg/kg per day in adult horses. The estimation was calculated on the basis of the superposition principle of the curves. The horizontal solid line represents the mean plasma gallium concentration after 15 days of administration (92.63 μg/L, equal to 99.68% of the steady state), and the horizontal dotted line represents the threshold concentration of 1 μM that we propose for beginning clinical trials on horses affected by navicular disease or other diseases characterised by bone remodelling and lysis.

The assertions on expected plasmatic levels following repeated oral administration are only indicative, because they have been estimated based on mathematical models rather than on experimental evidence and these require confirmation through *in vivo* testing. However, the validity of the superposition principle in calculating the concentrations resulting from chronic administration of oral Ga has been demonstrated in humans (Collery *et al.*, 1989), at least up to steady-state plasma concentrations of around 250 μg/L. Also, Chaffin *et al.* (2010) and Arnold *et al.* (2010) speculated in their studies that Ga plasma levels after oral administration of GaM in horses would follow the principle of superposition.

According to the present study, the administration of 10 mg/kg per day of oral GaN would achieve steady-state average concentrations of about 92.9 μg/L, corresponding to 1.33 μM.

On the basis of the existing literature (see Introduction), we hypothesise that prolonged exposure to plasma Ga levels of about 1 μM could lead to a decrease in bone catabolism, producing a therapeutic effect in animals affected by primary or secondary osteolytic disorders, also concurring with the considerations made by Arnold *et al.* (2010). From the standpoint of possible clinical applications of GaN in horses with navicular disease or other diseases characterised by bone remodelling and lysis, the dosage of 10 mg/kg per day administered orally over a period of at least 4 weeks would seem appropriate for initial clinical trials, because it would lead to plasma concentrations of 92.9 μg/L (1.33 μM) being achieved within 15 days (see Fig. 2).

None of the horses showed clinical signs or alterations in the haemato-biochemical parameters attributable to the administration of oral GaN, thus concurring with other toxicity studies (Martens *et al.*, 2010). Neither toxicity has been reported in any tested species at the oral dosage we intend to administer to horses for future clinical trials (10 mg/kg per day of GaN) nor at the plasmatic levels we estimate to reach in horses through repeated oral administration of 10 mg/kg per day (about 1.33 μM; Collery *et al.*, 1984; Vistelle *et al.*, 1989; Collery *et al.*, 1989, 2002). Ga is primarily toxic to the kidneys, because of the high peaks in Ga plasma levels (>200 μM) following intravenous administration (Krakoff *et al.*, 1979; Newman *et al.*, 1979; Bernstein, 1998). Instead, because of slow absorption rates, and therefore the high degree of Ga binding to protein, oral administration of Ga compounds is considered to be safe.

The addition of GaN to the barley ration did not affect palatability in the subjects tested during the pharmacokinetic study or in those used in the preliminary tests. We chose barley to mix with the GaN solution because crushed barley is well able to absorb liquids, thus minimising GaN loss in the feeding recipient. To the best of our knowledge, there should be no interaction between common horse foodstuffs, although no studies exist on this matter. The horses were fasted from 12 h before the experiment to 6 h afterwards, so that the ration would be consumed completely and rapidly, enabling a clear and well-readable absorption curve for a single administration to be obtained. From the standpoint of long-term repeated oral administration of GaN in future clinical trials, the customary feeding regimen for horses (without long fasting periods) should not affect the average plasma Ga concentration at steady state.

It should be mentioned here that other Ga compounds are currently being investigated, particularly gallium maltolate (GaM), a coordination complex of trivalent Ga cation with three maltolate ligands. GaM has shown itself to have a much higher oral bioavailability in humans if compared to Ga salts such as GaN or gallium chloride (Bernstein *et al.*, 2000). The kinetics of GaM have been recently studied in foals (Martens *et al.*, 2007, 2010; Chaffin *et al.*, 2010) and in adult horses (Arnold *et al.*, 2010). The results of those studies (Martens *et al.*, 2007; Arnold *et al.*, 2010) show marked differences in the kinetic parameters between foals and adult horses (foals: C_{max} = 1079 μg/L, $T_{½el}$ = 26.6 h, AUC = 40214 μg/L·h; adult horses: C_{max} = 280 μg/L, $T_{½el}$ = 51.5 h, $AUC_{0-\infty}$ = 20920 μg/L·h) following intragastric administration of 20 mg/kg of GaM (equal to 3.15 mg/kg of elemental Ga). This data disagreement could reflect differences in GaM absorption capability (or permeability) between the juvenile and adult gut and/or could be due to different Ga distribution volumes in foals and adult horses. However, the results of the aforementioned studies in connection with the findings of the present work suggest that oral bioavailability of GaM in horses is higher than that of GaN by about an order of magnitude.

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