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Comparative bioavailability study of two oral formulations of amoxicillin-clavulanic acid in healthy dogs

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Abstract

Background Amoxicillin-clavulanic acid combination (AMX-CA) is a widely used oral antibiotic for companion animals. In Thailand, various AMX-CA formulations are available. This study aimed to evaluate and compare the pharmacokinetic profiles and relative bioavailability of two AMX-CA formulations using a randomized, two-period, two-treatment crossover design in six healthy Beagle dogs. Each dog received a 250 mg AMX-CA tablet (formulation A or B) at a dosage of 20.5 ± 2.5 mg/kg, with a 7-day washout period between treatments. Blood samples were collected over a 24-h period post-administration, then AMX and CA concentrations were measured using LC–MS/MS. Bioequivalence was assessed based on the 90% confidence intervals (CI) for peak plasma concentration (C_{max}) and the area under the plasma concentration–time curve extrapolated to infinity ($AUC_{0-\infty}$), which required to fall within 80%-125%.

Results The relative bioavailability of formulation B was 76.5% for AMX and 72.7% for CA, compared to formulation A. Only CA's C_{max} met the bioequivalence criteria, while the CIs for $AUC_{0-\infty}$ and C_{max} of AMX and $AUC_{0-\infty}$ of CA were outside the acceptable range.

Conclusions Bioequivalence between the two formulations was not established, indicating that these formulations are not interchangeable.

Keywords Amoxicillin-clavulanic acid, Bioavailability, Bioequivalence, Dogs, Generic formulation, Interchangeability, LC–MS/MS

Background

Amoxicillin (AMX) belongs to β-lactam antibiotics that exhibit bactericidal activity against a wide range of gram-positive and gram-negative bacteria, excluding β-lactamase producing pathogens. On the other side, clavulanic acid (CA) is an irreversible β-lactamases inhibitor, which protects AMX from inactivation by β-lactamases, thereby expanding the antibacterial spectrum of AMX [1]. Oral AMX-CA is one of the most prescribed antimicrobials for companion animals. In dogs, this combination is used to treat skin and soft tissue infections like wounds, abscesses, cellulitis, and pyoderma caused by certain bacteria, including *Staphylococcus aureus*, *Streptococcus* spp., and *Escherichia coli* (*E. coli*). It is also effective

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against periodontal infections caused by both aerobic and anaerobic bacteria, as well as urinary tract infections caused by susceptible strains of *E. coli* [2].

Bioavailability describes the rate and extent to which an active drug or its metabolite enters the bloodstream and reaches its intended site of action. Bioequivalence is established when there is no discernible difference in bioavailability when administered at the same dosage under comparable conditions, with plasma drug concentrations serving as a surrogate measure. Products with equivalent rates and extents of drug absorption are considered therapeutically interchangeable in clinical practice [3]. Differences in bioavailability among products of the same active substances can be clinically significant, potentially leading to decreased efficacy and clinical failure [4]. Subtherapeutic antimicrobial exposure exerts selective pressure, allowing resistant bacteria to survive and proliferate while eliminating susceptible strains, ultimately leading to the dominance of resistant populations [5]. Inadequate AMX concentrations may promote bacterial adaptation, primarily by inducing β-lactamase production through mutations that enhance enzyme activity, overexpression due to regulatory site mutations, or gene amplification [6, 7]. Likewise, insufficient clavulanic acid may fail to fully inhibit β-lactamases, reducing susceptibility and increasing treatment failure. Subtherapeutic AMX-CA levels can further select for ESBL-producing bacteria [8], which spread resistance via plasmids [9], posing risks to both animal and human health [10]. The emergence of ESBL-producing bacteria has significantly limited treatment options in veterinary medicine [11]. Several studies have reported on AMX-CA resistance in companion animals [12–18]. A retrospective study on urinary tract infections (UTIs) in dogs in Thailand found 14–34% resistance to AMX-CA [12]. Another study on multidrug-resistant *E. coli* infections in dogs and cats at a veterinary teaching hospital reported approximately 45% resistance to AMX-CA [15]. Additionally, 37.4% of methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) isolates from dogs with cystitis exhibited resistance to AMX-CA [16]. While AMX-CA resistance in clinical isolates in Thailand appears lower compared to other antimicrobials like ampicillin and enrofloxacin [12, 15, 16], resistance has also been observed in healthy animals. A study on *E. coli* isolates from healthy animals found 100% resistance in cats and up to 20% in dogs. Additionally, ESBL-associated genes ($bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV}) were detected in 71.97% of isolates from cats and 21.69% from dogs, suggesting that *E. coli* from these animals may serve as reservoirs for antibiotic resistance [19]. Furthermore, the prevalence of acquired resistance in commensal bacteria highlights significant selective pressure from antimicrobial use in both animal and human populations [20–23].

From clinical observations, we noted several cases of treatment failure with oral AMX-CA, despite antimicrobial susceptibility testing indicating its susceptibility of the microorganism. Switching to a different drug brand has proven effective in some cases. This aligns with a previous report on fluconazole, which showed significant changes in plasma drug concentrations in dogs with clinical disease when the drug manufacturer was changed [24]. Additionally, a recent study on the quality of veterinary oral formulations of AMX-CA sampled from various countries found that one out of two formulations from Thailand failed to meet the assay content requirements for both AMX and CA [8]. These findings raise concern about the quality and the interchangeability of the AMX-CA products. Several oral AMX-CA veterinary products, sourced both from imports and local manufacturing, are available in Thailand. The small animal clinicians often choose oral formulation of AMX-CA products based on price, rather than considering interchangeability and bioequivalence criteria. However, bioequivalence studies are not a regulatory requirement for generic veterinary drug registration in many countries, including Thailand. Therefore, this study aimed to determine the bioequivalence and interchangeability of two AMX-CA oral formulations by evaluating the pharmacokinetic parameters and relative oral bioavailability in dogs.

Results

Animal procedure

All six dogs completed the study, with no adverse events observed after administration of either formulation A or formulation B at the mean AMX-CA dosage of 20.52 ± 2.50 mg/kg. The AMX reached a mean dosage of 16.42 mg/kg (range 14.23–19.14 mg/kg) and CA reached a mean dosage of 4.10 mg/kg (range 3.56–4.78 mg/kg).

LC–MS/MS method validation

The LC–MS/MS method successfully determined AMX and CA in dog plasma. The retention time of AMX and CA were 1.579 and 1.578 min, respectively. The retention time of AMP was 3.707 min. The endogenous matrix from plasma did not interfere with the peak of interest. The plasma calibration curves of both AMX and CA ranged from 0.05 µg/mL to 20 µg/mL, were reproducible over the tested concentration range, and exhibited linearity with average regression coefficient (R^2) > 0.99. The Lower limit of quantification (LLOQ) was 0.05 µg/mL. The intra-day and inter-day values for accuracy ranged from 95.73–12.40% and 96.50–107.23% (AMX) and 96.32–112.88% and 98.58–110.32% (CA). Both the intra-day and inter-day precisions were < 6.23% for AMX and < 6.03% for CA at three quality control levels (0.15, 7.5 and 15 µg/

mL). The extraction recoveries of AMX were between 85.93% and 92.76%, while those of CA were between 82.17% and 88.41%. The method used in this study was in accordance with the standard requirements of bioanalysis method validation [25].

Plasma concentration of AMX and CA

Semi-logarithmic plots of the plasma concentration–time curves of AMX and CA after a single oral administration are shown in Figs. 1 and 2. Peak plasma concentrations of AMX were observed at 1.5 h for formulation A and 1.5–2 h for formulation B. AMX was quantified for 12 h after oral dosing, and plasma concentration of CA reached the peak within 1–1.5 h for both formulations. CA concentrations in most of the samples were measurable up to 4 h post-dose. At the 8-h timepoint, only two dogs per group (2/6 dogs received formulation A and 2/6 dog received formulation B) showed

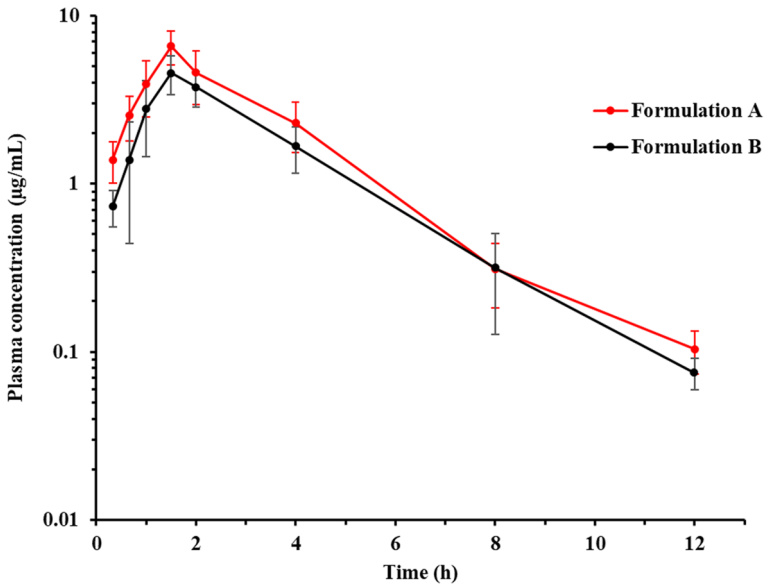

Fig. 1 The plasma concentration–time curves of AMX in dogs received a single oral administration of AMX-CA 250 mg formulation A and formulation B ($n=6$)

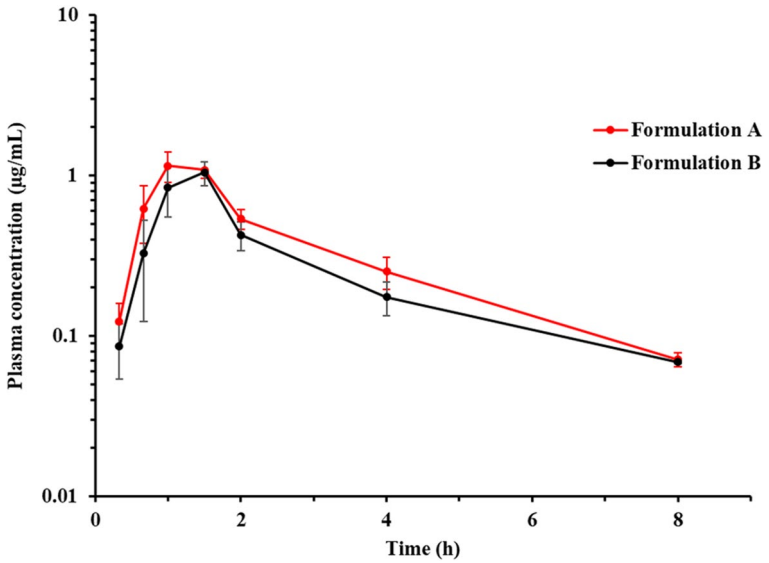

Fig. 2 The plasma concentration–time curves of CA in dogs received a single oral administration of AMX-CA 250 mg formulation A and formulation B (all timepoints: $n=6$, at 8 h: $n=2$)

Table 1 Pharmacokinetic (PK) parameters of AMX after single oral administration AMX-CA 250 mg either formulation A or formulation B in dogs (Mean ± SD, n = 6)

PK Parameter	Unit	AMX-CA 250 mg formulations	
		Formulation A	Formulation B
C_{max}	μg/mL	6.58 ± 1.37[a]	4.79 ± 0.97
T_{max}	h	1.50 ± 0.00	1.67 ± 0.24
$t_{1/2}$	h	1.80 ± 0.15	1.82 ± 0.19
k_{el}	1/h	0.39 ± 0.03	0.39 ± 0.04
AUC_{0-12}	μg·h/mL	20.31 ± 5.33	15.28 ± 2.89
$AUC_{0-\infty}$	μg·h/mL	20.59 ± 5.37	15.48 ± 2.92

C_{max} peak plasma concentration, T_{max} time to achieve peak concentration, $t_{1/2}$ elimination half-life, k_{el} elimination rate constant, AUC_{0-12} area under the plasma concentration versus time curve from 0 to 12 h (the last measurable timepoint), $AUC_{0-\infty}$ area under the plasma concentration versus time curve extrapolated to infinity

[a] significant difference between columns ($p < 0.05$)

Table 2 Pharmacokinetic (PK) parameters of CA after single oral administration AMX-CA 250 mg either formulation A or formulation B in dogs (Mean ± SD, n = 6)

PK Parameter	Unit	AMX-CA 250 mg formulations	
		Formulation A	Formulation B
C_{max}	μg/mL	1.22 ± 0.14	1.10 ± 0.15
T_{max}	h	1.08 ± 0.19	1.33 ± 0.24
$t_{1/2}$	h	1.55 ± 0.33	1.21 ± 0.35
k_{el}	1/h	0.47 ± 0.10	0.61 ± 0.13
AUC_{0-4}	μg·h/mL	2.19 ± 0.30 [a]	1.72 ± 0.32
$AUC_{0-\infty}$	μg·h/mL	2.80 ± 0.52 [a]	2.06 ± 0.51

C_{max} peak plasma concentration, T_{max} time to achieve peak concentration, $t_{1/2}$ elimination half-life, k_{el} elimination rate constant, AUC_{0-4} area under the plasma concentration versus time curve from 0 to 4 h (the last measurable timepoint), $AUC_{0-\infty}$ area under the plasma concentration versus time curve extrapolated to infinity

[a] significant difference between columns ($p < 0.05$)

measurable plasma CA concentrations. For both formulations, the plasma concentrations of AMX and CA were below the LLOQ (0.05 μg/mL) at 12 h and 4–8 h, respectively.

Pharmacokinetic parameters

The pharmacokinetic parameters for each drug formulation based on active substance, AMX and CA, are presented in Tables 1 and 2. Pharmacokinetic parameters of AMX from the two formulations showed that the mean C_{max}, AUC_{0-12} and $AUC_{0-\infty}$ of formulation B were lower than those of formulation A. However, only the C_{max} values were significantly different ($p < 0.05$). The pharmacokinetic parameters of CA (mean AUC_{0-4} and $AUC_{0-\infty}$) were statistically different with a greater value for the formulation A ($p < 0.05$).

Geometric mean ratios of natural log-transformed $AUC_{0-\infty}$ and C_{max} and their 90% CI and RBA of AMX and CA are presented in Table 3. The mean RBA (AMX and CA) of formulation B to that of formulation A were 76.50% and 72.70%.

Discussion

In this study, all dogs received AMX-CA formulation as a single tablet without splitting or crushing, to minimize the impact of changes in disintegration and dissolution. The average dosage of AMX-CA was 20.52 ± 2.50 mg/kg body weight, within the current recommended dose between 12.5–25 mg/kg body weight every 12 h [26]. Both formulations showed a similar pattern of plasma drug concentration–time curve, consistent with previous reports in dogs [27] and cats [28]. This pattern is characterized by rapid absorption of AMX and CA after oral administration, with peak plasma levels reached within 1 to 2 h, followed by rapid decline and becoming unmeasurable within 12 h for AMX and 4 to 8 h for CA. However, Moczarnik et al. reported later absorption times, with

Table 3 Bioequivalence analysis for natural log- transformed $AUC_{0-\infty}$ and C_{max} of two AMX-CA oral formulations (n = 6)

Substance	PK Parameter	Unit	Geometric mean		Geometric mean ratio (90% CI)
			Formulation A	Formulation B	
AMX	C_{max}	μg/mL	6.44	4.68	0.73 (0.68–0.76)
	$AUC_{0-\infty}$	μg·h/mL	19.90	15.22	0.77 (0.68–0.89)
	RBA	%	76.50		
CA	C_{max}	μg/mL	1.21	1.09	0.90 (0.86–0.94)
	$AUC_{0-\infty}$	μg·h/mL	2.76	2.01	0.73 (0.69–0.77)
	RBA	%	72.70		

C_{max} peak plasma concentration, $AUC_{0-\infty}$ area under the plasma concentration versus time curve extrapolated to infinity. *RBA* relative oral bioavailability, *CI* confident interval

their results indicating a T_{max} of 2.1 to 2.45 h (AMX) and 1.26 to 1.44 h (CA) [29]. This difference may be attributed to the food effect, as dogs were administered feed along with the drug. Limited studies are available on the pharmacokinetics of the AMX-CA in companion animals. In an earlier investigation, beagles were given AMX-CA orally at a dose of 12.5 mg/kg. The study demonstrated that AMX and CA were sufficiently absorbed and distributed, allowing for the prediction of efficacy against infections caused by β-lactamase-producing bacteria [30]. Significant individual variation in CA absorption has been reported in both humans [31, 32] and dogs [27] following oral administration of the AMX-CA combination. However, AMX absorption appears to be less variable, possibly due to a saturable absorption process in which high AMX doses inhibit the concurrent absorption of CA [27]. In our study, individual variability in absorption was assessed using the coefficient of variation (CV%) for AUC and C_{max} of both substances from formulations A and B. The CV% for AMX's AUC and C_{max} were 29.19% and 27.32%, respectively, whereas for CA, the corresponding values were 20.83% and 14.43%. These findings contrast with previous studies, as our results indicate that AMX exhibits greater variability than CA. This discrepancy may be attributed to differences in dosage, as the previous study in dogs used a higher dose (AMX: 33.3 ± 4.17 mg/kg, CA: 8.30 ± 1.03 mg/kg) [27]

According to the bioequivalence study guidelines for veterinary medicinal products with systemic action, generic drugs should be compared to the original drug in healthy target animals [3]. Our study was conducted in 2023, by which time the original product had not been marketed in Thailand for a few years. The imported generic product approved worldwide was used as reference for comparison with the generic one. Both are registered veterinary medicines commonly used in veterinary clinics and hospitals in Thailand. Regulatory agencies like the FDA and EMA suggest a sample size of at least 8–12 in crossover designs for pivotal BE studies [3, 33]. However, due to limitations in animal availability, our study followed the precedent set by similar published prospective pharmacokinetic studies with comparable sample sizes [29, 34, 35]. The power analysis of the sample size used in this study was calculated using G*Power software version 3.1.9.7 (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany) and demonstrated a statistical power exceeding 80%, indicating sufficient sensitivity to detect meaningful differences. However, this calculation alone may not fully ensure statistical robustness for BE studies. For future comprehensive BE studies, we recommend determining the sample size through a complete power analysis or increasing it to meet national and international regulatory guidelines, ensuring greater statistical reliability and generalizability.

The key parameters for bioequivalence assessment are AUC and C_{max}. AUC reflects the overall absorption of a drug, while C_{max} and T_{max} collectively indicate the rate of absorption [3, 33]. The data obtained from pharmacokinetic analysis of AMX indicated that formulation A exhibited greater absorption than formulation B. Although only the C_{max} values were significantly different ($p < 0.05$), the mean AUC_{0-12} and $AUC_{0-\infty}$ of formulation B were lower than those of formulation A. The elimination of AMX from the two formulations appeared to be similar. For CA, formulation A also showed greater absorption, as evidenced by statistically significant higher mean AUC_{0-4} and $AUC_{0-\infty}$ values compared to formulation B ($p < 0.05$). However, the elimination rate of CA from formulation A, as indicated by $t_{1/2}$ and k_{el}, seemed to be slower than that of formulation B. These findings suggest that the differences in pharmacokinetics for both AMX and CA may stem from varying absorption rates. The variability in the elimination may also be a contributing factor for CA.

Bioequivalence between two veterinary drugs is established if the upper and lower limits of the 90% confidence interval (CI) of the generic to brand ratio for AUC and C_{max} fall within the range of 80–125% [3, 33]. Our results showed that the 90% CI range between 80–125% was observed only for the C_{max} of CA, while the 90% CI for the respective mean ratios for $AUC_{0-\infty}$ and C_{max} of AMX, as well as $AUC_{0-\infty}$ of CA, did not fall within the acceptable range. The average RBA of formulation B to that of formulation A was 76.50% and 72.70% for AMX and CA, respectively. These results indicate unequal drug exposure between the two formulations. Therefore, bioequivalence between the two formulations could not be established. Our findings align with a previous bioequivalence study of AMX-CA oral formulations in dogs, which demonstrated no equivalence between the reference product AMX-CA and the locally produced generic product in Mexico. That study reported a relative oral bioavailability of 68.44% for the generic product [36]. Several factors in the clinical setting can affect the bioavailability of drugs, including food, disease, and concurrent medications [37]. However, in the experimental design for bioequivalence studies, the primary focus is on the effects of the drug product. The bioequivalence discrepancy of the drug may be due to the substandard quality of the product, including the amount and quality of the active substance or the effect of excipients. Oral formulation is typically a compressed tablet, requiring disintegration and dissolution before absorption and distribution in the body. The homogeneity of excipients is

a crucial pharmaceutical factor that can impact the efficiency of these processes and the bioavailability of the drug [38]. Variations in excipients, tablet composition, or manufacturing processes can impact drug dissolution and subsequent absorption [39, 40]. The lack of in vitro dissolution data represents a limitation of our study. To enhance the evaluation of generic formulations, future research should incorporate comparative in vitro dissolution testing alongside pharmacokinetic assessments. This approach would provide deeper insights into whether differences in bioavailability stem from formulation-related factors affecting drug release and absorption. For the active substances, it is difficult to differentiate between substandard quality due to non-compliance with good manufacturing practices and post-production degradation due to improper storage [8]. Some formulations may initially be of good quality but may later deteriorate. Degradation can occur in both AMX and CA, with a higher incidence of degradation observed for CA, which can be attributed to its lower stability compared to AMX [41].

As time-dependent antibiotics, their antibacterial efficacy is determined by the period during which plasma concentrations exceed the minimal inhibitory concentration (T > MIC) [42, 43]. The optimal duration for maintaining plasma concentrations above the MIC has varied across studies; however, a common assumption for β-lactams is that drug concentrations should exceed the MIC for at least 40–50% of the dosing interval [44]. If the MIC values are established at 0.25 μg/mL for AMX and 0.12 μg/mL for CA, based on the susceptible breakpoint values determined by the Clinical and Laboratory Standards Institute (CLSI) for *Escherichia coli* causing skin and soft tissue infections in dogs [45], the concentration of AMX in the present study remained above the MIC for 9 h and 8.7 h for formulation A and formulation B, respectively. The T > MIC of AMX is more than 50% of the typically recommended 12-h dosage interval for both formulations. Meanwhile, the concentration of CA remained above the MIC for 6.5 h and 5.8 h, accounting for 54.17% and 48.30% of the 12-h dosing interval, for formulation A and formulation B, respectively. As T > MIC of AMX and CA for both formulations were within the target values, clinical efficacy would be expected for both formulations in the treatment of AMX-CA susceptible microorganisms. However, the studied formulations are not interchangeable because bioequivalence could not be demonstrated. These findings align with prior research on the pharmacokinetics of two parenteral cephalexin formulations in dogs, suggesting that non-bioequivalent commercial formulations may not be interchangeable, even when similar plasma profiles and no statistically significant differences in pharmacokinetic parameters or T > MIC are observed [34]. Furthermore, as formulation B maintained CA levels above the MIC for less than 50% of the dosing interval, it may reduce therapeutic efficacy and increase the risk of treatment failure against β-lactamase-producing pathogens, ultimately heightening the potential for antimicrobial resistance. However, these pharmacokinetic/pharmacodynamic integrations must be interpreted with caution, as the MIC values are based on susceptible breakpoint values derived from examining various MIC distribution data [45], which may vary geographically in isolates susceptibility. Moreover, this study investigated the pharmacokinetics of drugs in healthy animals. It has been previously reported that the pharmacokinetics of AMX-CA differ significantly in critically ill dogs compared to normal dogs, exhibiting much higher interindividual variability and lower systemic clearance [46].

In Thailand, BE studies are not mandatory for the registration of generic veterinary drugs, which differs from regulatory frameworks in the United States (FDA) and the European Union (EMA). The FDA's Center for Veterinary Medicine (CVM) requires BE studies for the approval of generic veterinary drugs under the Abbreviated New Animal Drug Application (ANADA) process. According to FDA guidelines, a generic drug must demonstrate BE to an approved reference product to ensure comparable absorption and efficacy [47]. Similarly, the EMA mandates BE studies as part of the marketing authorization process for veterinary generics, with exemptions granted only when justified by alternative scientific evidence [48]. In contrast, Thailand's generic veterinary drug registration process primarily relies on pharmaceutical equivalence (e.g., same active ingredient, strength, and dosage form), without requiring in vivo BE studies. This regulatory gap raises concerns regarding the clinical efficacy and interchangeability of generic veterinary drugs, particularly for antimicrobial agents, where inconsistent plasma drug levels could lead to treatment failure or contribute to antimicrobial resistance (AMR). Our study highlights the importance of monitoring the quality of generic veterinary drugs and may contribute to policy discussions on enforcing BE studies in Thailand. Strengthening regulations to align with international standards would enhance the quality, efficacy, and safety of veterinary generics, ensuring compliance with international benchmarks and supporting sustainable antimicrobial stewardship.

Conclusions

This study compared the pharmacokinetic profiles of two oral formulations of AMX-CA, a brand generic product and a locally produced generic product. The relative oral bioavailability of the locally produced product was 76.50% for AMX and 72.70% for CA. Bioequivalence

between both formulations could not be demonstrated, as the lower 90% CI failed to fall within the accepted range of 80–125%. These findings suggest that the formulations are not interchangeable. Our results highlight the importance of considering product quality in drug use, as it may impact treatment effectiveness and contribute to increasing antimicrobial resistance. Further research and monitoring are necessary to gain a more comprehensive understanding and develop appropriate strategies to address these issues.

Methods

Drugs and chemicals

Analytical standards of amoxicillin trihydrate, potassium clavulanate and ampicillin trihydrate were purchased from Sigma-Aldrich, USA. Acetonitrile (LRC Labscan, Thailand) of liquid chromatography-mass spectrometry grade and ammonium formate (Sigma-Aldrich, Germany) of analytical grade were used. Two generic veterinary products containing 250 mg of AMX-CA were included in the study: an imported generic product (referred to as formulation A) and a locally produced generic product (referred to as formulation B). Since this study has no conflict of interest with the products, the commercial names of the products are not disclosed in this article.

Animals and experimental design

Six healthy male beagle dogs with an average body weight of 12.3 ± 1.46 kg, and aged 8 ± 3.29 years, were obtained from the demonstration animal colony of the Department of Obstetrics Gynecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University. They had no recent antimicrobial treatment within the two months prior to the study. All dogs were confirmed to be clinically healthy by physical and clinical biochemical examinations. The dogs were housed in individual kennels with ambient temperature (28–33 °C) and humidity (40–70%). They were fed drug—free commercial dry feed twice a day and given water ad libitum throughout the study period.

A two-period two-treatment crossover design with a 7-day washout period was used in this study. Six dogs were randomly allocated to the two crossover study groups. For each study period, a single dose (one tablet per dog) of either AMX-CA 250 mg formulation A or formulation B was administered. The average AMX-CA dosage was 20.5 ± 2.5 mg/kg of body weight. The drug was given 2 h before feeding. The tablets were administered to the animals on the base of the tongue. Three milliliters of water was given after each oral administration, and the mouth was checked to ensure that the tablet had been swallowed.

The animal experiment was conducted at the Veterinary Student Training Center, Nakorn Pathom, Faculty of Veterinary Science, Chulalongkorn University. All animal experiments were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Science, Chulalongkorn University (protocol code: 2231063).

Sample collection and processing

Three milliliters of blood samples were collected from each dog through an intravenous catheter from a cephalic vein. Blood samples were collected at 10 timepoints before and after drug administration; 0, 20, 40 min and 1, 1.5, 2, 4, 8, 12 and 24 h. Blood samples were transferred to heparinized tubes and centrifuged at 1000×*g* for 15 min at 4 °C, then plasma was harvested in sterile cryovials and stored at −80 °C until analysis.

For drug extraction, a 200 µL of plasma sample was added to a 1.5 mL microcentrifuge tube and spiked with 20 µL of internal standard solution containing 10 µg/mL ampicillin (AMP). Afterward, 600 µL of acetonitrile was added and vortex-mixed for 1 min. The mixture was centrifuged at 15,000×*g* 4 °C for 15 min. The supernatant solution was collected and passed through a 0.22 µm syringe filter into a sampler vial and subjected to LC–MS/MS.

LC–MS/MS procedure

Plasma concentrations of AMX and CA were measured simultaneously by LC–MS/MS. The system was equipped with a Shimadzu LCMS-8045 (Shimadzu, Japan) with Lab solution version 5.82 SP1 software. Separation was achieved using Shim-pack GIST-HP C18 column (150×2.1 mm, 3 µm; Shimadzu, Japan) coupled with a Shim-pack GIST-HP(G) C18 guard column (10×12.1 mm, 3 µm; Shimadzu, Japan). The column was maintained at a temperature of 40 °C. The mobile phase consisted of a binary gradient of 10 mM ammonium formate in water (mobile phase A) and acetonitrile (mobile phase B). The gradient condition was as follows: 0–2.8 min, from 0 to 95% B; 2.8–4.0 min, maintained at 95% B; 4.0–4.1 min, 95% to 0% B and 4.1–8.0 min, maintained at 0% B. The flow rate was 300 µL/min. The extracted samples were maintained in an auto-sampler at 15 °C. The injection volume was 20 µL. The analytes were detected with a triple quadrupole mass spectrometer equipped with an electrospray ionization source that was operated using positive ionization mode for AMX and AMP and negative ionization mode for CA. The mass spectrometer was operated under the multiple reaction monitoring (MRM) mode. The following transitions were

used: AMX: m/z 366 > 144 and 207.9; CA: 198.4 > 136.1 and 108.2; AMP (IS): m/z 350 > 106.2, 160 and 192.1.

The calibration standard concentrations of AMX and CA were prepared by spiking the working standard solution into blank plasma to yield final concentrations of 0.05, 0.5, 1, 2.5, 5, 10 and 20 µg/mL. Quantitative determination was performed by internal standard calibration. Linear regression was applied with a weighing factor of 1/x. The LC–MS/MS method was validated following bioanalytical method validation guidelines, covering key parameters such as selectivity, linearity, lower limit of quantification, accuracy, precision, and recovery [25].

Pharmacokinetic analysis and statistical analysis

Plasma concentrations of AMX and CA with respect to time were pharmacokinetically analyzed using a non-compartmental model with the STATA® software version 15 (StataCorp LLC, USA). The following PK parameters were calculated: peak plasma concentration (C_{max}), time to achieve peak concentration (T_{max}), elimination rate constant (k_{el}), elimination half-life ($t_{1/2}$), area under the plasma concentration versus time curve from 0 to the last measurable timepoint (AUC_{0-t}), area under the plasma concentration versus time curve extrapolated to infinity ($AUC_{0-\infty}$). AUC was determined using trapezoidal rule. Mean values for each of these PK parameters between the two drug formulations were compared with Student t-test using SPSS 22.0 statistic software (IBM Co., Chicago, Illinois, IL, USA). The results were presented as mean ± standard deviation (SD). Differences were considered statistically significant when p-value was lower than 0.05. The relative bioavailability (RBA) was calculated as percentage of $AUC_{0-\infty}$ ratio ($AUC_{0-\infty}$ of formulation B/$AUC_{0-\infty}$ of formulation A). Bioequivalent assessments were based on log-transformed of $AUC_{0-\infty}$ and C_{max}. Geometric means, geometric mean ratio and the 90% confidence interval (CI) of the geometric mean ratio were calculated for the transformed pharmacokinetic parameters. Bioequivalence was concluded when the geometric mean ratio and 90% CI for $AUC_{0-\infty}$ and C_{max} fell within the limits of 80–125% [3].

Abbreviations

AMP	Ampicillin
AMX	Amoxicillin
AMX-CA	Amoxicillin-clavulanic acid combination
AUC_{0-t}	Area under the plasma concentration versus time curve from 0 to the last measurable timepoint
$AUC_{0-\infty}$	Area under the plasma concentration versus time curve extrapolated to infinity
CA	Clavulanic acid
CI	Confidence intervals
C_{max}	Peak plasma concentration
ESBL	Extended-spectrum β-lactamase
k_{el}	Elimination rate constant
LC–MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LLOQ	Lower limit of quantification
R^2	Regression coefficient
SD	Standard deviation
T > MIC	The period during which plasma concentrations exceed the minimal inhibitory concentration
$t_{1/2}$	Elimination half-life
T_{max}	Time to achieve peak concentration
RBA	Relative oral bioavailability

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Authors' contributions

KV and NS contributed to the study design, conducted experiments, analyzed data, and drafted the manuscript. KP and PC participated in animal experiments. SN and HC contributed to the study design and provided critical revisions to the manuscript. All authors have read and agreed to the final version of the manuscript.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The animal experiments were carried out in compliance with the ARRIVE guidelines and ethical standards of the University. This study was performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Science, Chulalongkorn University (protocol code: 2231063).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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