Feasibility of interspecies extrapolation in determining the bioequivalence of animal products intended for intramuscular administration

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To examine the validity of extrapolating parenteral product bioequivalence determinations across target animal species, the relative bioavailability of two injectable formulations of ampicillin trihydrate (Polyflex^R, a water-based suspension, and Ampi-kel 10^R, an oil-based suspension) was examined in calves, sheep and swine. Employing products recognized to be bioinequivalent provided an opportunity to explore potential species-by-formulation interactions. As compared with Polyflex^R, Ampi-kel 10^R exhibited lower area under the curve (*AUC*) estimates but higher peak concentrations in all target animal species. Nevertheless, marked interspecies differences were noted in the width and bounds of the confidence intervals about the differences in treatment means. Potential physiological and physico-chemical reasons for these findings are discussed.

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INTRODUCTION

To obtain marketing approval, generic formulations must demonstrate bioequivalence to the marketed pioneer product in every target animal species included on the pioneer product label. This policy necessitates that generic drug sponsors submit multiple bioequivalence investigations, each employing approximately 20 animals. Inherent in this requirement is the assumption that product bioequivalence cannot be extrapolated across target animal species.

It is widely recognized that serum drug concentrations can vary across animal species because of interspecies differences in clearance, volume of distribution, and in gastrointestinal (GI) physiology (Kalarli, 1995; Riviere *et al.*, 1997). Parenteral drug absorption may likewise vary as a result of differences in the physico-chemical properties of the injection site (Baggot & Brown, 1998). Furthermore, interspecies differences are known to occur in metabolic endproducts, protein binding and enterohepatic recycling (Riond *et al.*, 1990; Davies & Morris, 1993; Short, 1993). Nevertheless, while two animal species may exhibit dissimilar serum concentration/time profiles, they may show similar effects of product formulation on product drug absorption characteristics.

To date, the conditions promoting species-by-formulation interactions have not been well described. While inconsistencies in oral product relative bioavailability have been reported (Aoyagi *et al.*, 1984; Ogata *et al.*, 1984; Kaniwa *et al.*, 1991) it is uncertain whether or not similar interspecies differences occur with parenteral dosage forms. Therefore, to explore this possibility, we selected ampicillin trihydrate; a compound labelled for use in multiple veterinary species and marketed in several bioinequivalent parenteral formulations (Nouws *et al.*, 1982). The water-soluble Polyflex^R suspension is currently marketed in the US while the oil-based Ampi-kel 10^R suspension is a European formulation. Testing bioinequivalent products provided an assurance that formulation factors (such as *in-vivo* dissolution) rather than physiological attributes (such as membrane permeability) would be the rate-limiting elements dictating the rate and/or extent of drug absorption. As these two products are known to be bioinequivalent, the interspecies comparisons focused on the width and bounds of the confidence intervals about the difference in product area under the curve (*AUC*) estimates and peak plasma concentrations (CMAX).

MATERIALS AND METHODS

Study design

The investigation employed six mature animals of each species (bovine, porcine, ovine) weighing (mean ± SEM) 152.2 ± 6.0, 43.11 ± 1.0, and 49.0 ± 2.28 kg, respectively. Animals were acclimatised indoors for at least 1 week prior to the beginning

of the experiment and remained indoors until study completion. They were given a health examination upon arrival, provided with water *ad libitum*, and fed appropriate basal diets. Weights were measured from 24 to 48 h prior to each dosing (periods 1 and 2) to determine the appropriate injection volume.

The two commercially available formulations of ampicillin trihydrate were

- Ampi-kel 10^R, 100 mg ampicillin base/mL in an oily suspension Kela Lab. NV, Belgium.
- Polyflex^R, 100 mg ampicillin/mL aqueous suspension, Bristol Veterinary Products, USA.

The relative bioavailability of the two formulations was tested in each target animal species using a two-treatment, two-sequence, two-period crossover study design. During period 1, three animals of each species received an intramuscular (i.m.) injection of Ampi-kel 10^R (sequence 1) and three animals were injected with Polyflex^R (sequence 2). The treatments were switched during period 2. A 5-day washout interval separated study periods.

Ampicillin trihydrate was administered via i.m. injection to calves and swine based upon the approved label dose (11 mg/kg body weight for calves; 6.6 mg/kg body weight for swine). As ampicillin trihydrate is not currently approved for use in sheep, an extrapolated i.m. dose (10 mg/kg body weight) was administered. A single i.m. dose was injected deep into the semitendinosus muscle of calves, sheep, and pigs. The drug was administered to the identical side of the body during periods 1 and 2.

Serial venous blood samples (10 mL) were collected from the jugular veins (calves, sheep) or through a catheter inserted into the anterior cava (Brochat *et al.*, 1989). $EDTAK_2$-containing plastic syringes were used to collect blood prior to dosing (hr zero) and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 30, 36, and 48 h postdose. The plasma was separated by centrifugation ($1300 \times g \times 15$ m at 4 °C), harvested, and stored at −50 °C until analysis.

Product bioavailability was characterized on the basis of the *AUC* (linear trapezoidal method) and CMAX. As the terminal elimination rate constant could not be reliably estimated in several animals, *AUC* values were limited to the area estimated from hour zero to the time of the last quantifiable drug concentration.

Analytical method

The activity of ampicillin in the plasma was measured using a bioassay assay procedure (Dey & White, 1993). Residue concentrations were quantitated as ampicillin base. The test microorganism, *Micrococcus luteus* (ATCC 9341a), was grown in agar Antibiotic Medium 2. Using a minimal count of 1×10^6 microorganisms, the analytical method was found to be linear from 0.0125 to 0.2000 μg ampicillin base/mL for all three target animal species. The lower limit of quantitation was 0.0125 μg/mL. Nine replicates of each plasma and reference sample were assayed.

Statistical evaluation

Interspecies comparisons. Bioavailability parameter estimates were dose-normalized to 6.6 mg/kg. Interspecies comparison of parameter values was based upon two separate sets of analysis of variance (ANOVA) procedures (SAS, Release 6.12). For the pooled dataset, the statistical model included effects associated with species, subject nested within species, treatment, period and treatment-by-species interaction. The mean square error for the latter effect was tested against the residual error term to determine its statistical significance. Subject nested within species was the error term for testing the significance of the species effects. Appropriateness of this model was confirmed by using the Q option within SAS and specifying subject nested within species as the random effect term.

Species effects were also examined within the individual treatment groups. When statistically significant species effects were observed, pairwise comparisons were generated using the studentized t distribution to test the null hypothesis that LSMean[i] = LSMean[j], where i and j represent the Ln-transformed parameter means associated with species i and j, respectively.

To determine the power associated with a statistical test, the detectable differences were calculated according to the equation (Winer, 1971):

$$D = \{S_t\sqrt{3/n}\} \times (\delta_{\alpha/2(\nu)})$$

where D = the detectable difference at $\alpha = 0.025$ and $\beta = 0.20$, $\delta_{\alpha/2(\nu)}$ = the noncentrality parameter for $\alpha/2$ and ν degrees of freedom[1], $\nu = 2(n-1)$, $1 - \alpha/2 = 0.975$, $\beta = 0.20$, S_t = the square root of the pooled variances and n = the total number of observations included in the comparison.

Detectable differences were then expressed as a percent value relative to the grand mean from the corresponding ANOVA.

Product bioequivalence. Product bioequivalence was evaluated separately within each target animal species. As equivalence was handled as a within-species comparison, dose normalization was not needed. The within-species ANOVA model included effects associated with sequence, subject nested within sequence, period and treatment. The confidence intervals about the difference in treatment means were calculated in accordance with the 1996 CVM Bioequivalence Guidance (Anon 1). For the purpose of this investigation, Polyflex^R was considered the reference product.

Simulations

All simulations were conducted using the WINNONLIN pharmacokinetic software program (Version 1.5). Model parameters are detailed in the text.

[1] It should be noted that if noncentral t tables are not available, the value corresponding to $\delta_{a/2(\nu)}$ can be determined as the sum of $t_{\alpha/2(\nu)} + t_{0.2(\nu)}$ where the t-values represent the probabilities associated with a one-tailed test.

RESULTS

Interspecies comparison

The ampicillin plasma concentration vs. time profiles following the administration of Ampi-kel 10^R or Polyflex^R to calves, swine and sheep are provided in Fig. 1a (Ampi-kel 10^R) and 1b (Polyflex^R). All plasma concentrations have been normalized to a 6.6-mg/kg dose. The corresponding dose-normalized *AUC* and CMAX values are provided in Table 1. Within-product interspecies ratios are provided in Table 2.

Based upon the dose-normalized data (pooled across treatments), less than a 10% interspecies difference was detected in the extent of ampicillin bioavailability ($P = 0.092$). However, statistically significant interspecies differences were observed in rate of absorption ($P = 0.036$). Regardless of formulation, swine exhibited the largest CMAX values while the lowest values were consistently seen with sheep. Neither parameter was associated with a statistically significant species-by-formulation interaction ($P = 0.344$ and 0.458). However, failure to demonstrate significance was, at least in part, attributable to low statistical power (detectable differences were 15.8 and 25.6% for *AUC* and CMAX, respectively).

Given the low power associated with the test for species-by-formulation interaction, we chose to explore interspecies differences in CMAX values within the individual treatments. Although the magnitude of interspecies differences was greatest with Ampi-kel 10^R, statistically significant differences were only observed for the Polyflex^R treatment. Again, this apparent inconsistency was attributable to the respective power of the statistical tests. While interspecies differences as small as 21% could be detected in the Polyflex^R treatment group, a difference of no less than 33% could be detected as significant for the Ampi-kel 10^R comparison.

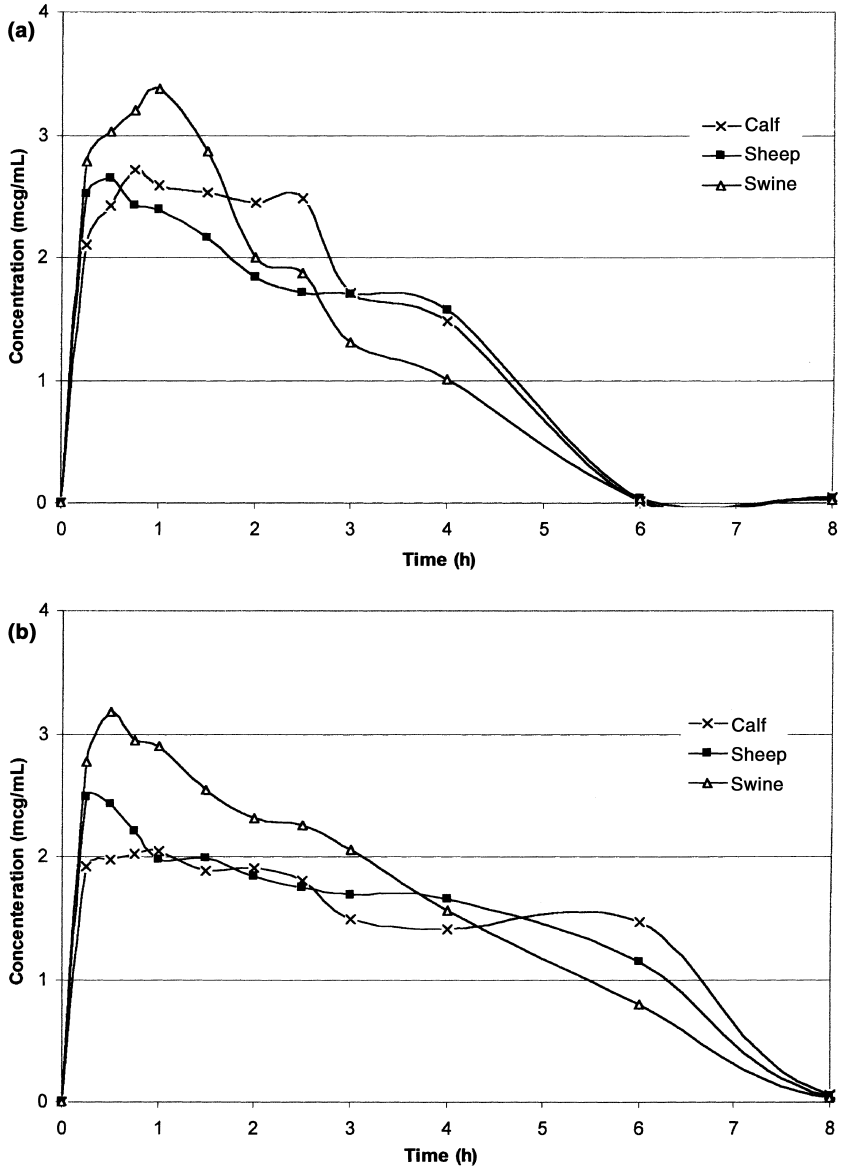

Fig. 1. Mean plasma ampicillin concentration vs. time profiles for calves, sheep and swine. All concentrations have been normalized to a 6.6-mg/kg dose. (a) Following administration of Ampi-kel 10^R (11 mg/kg in calves, 10 mg/kg in sheep, 6.6 mg/kg in swine). (b) Following the administration of Polyflex^R. In all cases, the administered dose of ampicillin trihydrate was 11 mg/kg for calves, 10 mg/kg for sheep, and 6.6 mg/kg for swine.

Table 1. Dose-normalized *AUC* and CMAX values

Variable	Mean	SD	Between-subject %CV
Ampi-kel 10^R			
Calf *AUC* (μg × *η*/mL)	10.44	0.37	3.5
Sheep *AUC*(μg × h/mL)	9.46	1.16	12.2
Swine *AUC*(μg × h/mL)	9.60	0.86	8.9
Calf CMAX (μg/mL)	3.41	0.62	18.2
Sheep CMAX (μg/mL)	2.89	0.45	15.5
Swine CMAX (μg/mL)	3.65	0.95	26.0
Polyflex^R			
Calf *AUC* (μg × h/mL)	12.05	0.85	7.0
Sheep *AUC* (μg × h/mL)	11.62	1.72	14.8
Swine *AUC* (μg × h/mL)	12.57	1.42	11.3
Calf CMAX (μg/mL)	2.62	0.42	16.1
Sheep CMAX (μg/mL)	2.59	0.37	14.2
Swine CMAX (μg/mL)	3.25	0.33	10.1

Table 2. Ratio of *AUC* and CMAX values across target animal species

		Interspecies comparison		
Product	Metric	Calf/sheep	Calf/swine	Sheep/swine
Ampi-kel 10^R	*AUC*	1.10	1.09	0.99
.	CMAX	1.18	0.93	0.79
Polyflex^R	*AUC*	1.04	0.96	0.92
	CMAX	1.01	0.81*	0.80*

* Statistically significantly different parameter estimates for the two species being compared ($P < 0.05$).

Within-species bioequivalence comparison

The confidence intervals about the differences in treatment means were evaluated for *AUC* and CMAX. For *AUC*, the intervals in both calves and sheep were unexpectedly narrow because of the very low intrasubject variability (as reflected by the residual error). In comparison, the confidence bounds for *AUC* in swine were lower and the interval wider than those observed in the other two species (Table 3, Fig. 2).

With regard to CMAX, the confidence intervals were substantially wider than were those estimated for *AUC*. Although the confidence bounds for CMAX were nearly identical for swine and sheep, they were markedly different for cattle. In fact, while the CMAX confidence bounds for sheep and swine were contained within the bioequivalence limits of 0.80–1.25, this was not the case for cattle.

In sheep, statistically significant sequence effects were observed for CMAX ($P = 0.006$). Statistically significant sequence ($P = 0.045$) and period ($P = 0.003$) were observed for *AUC*.[2] Statistically significant sequence effects were not observed in the other two species. As significant sequence plus period effects indicate the possibility of unequal first order residuals (i.e. carryover effects), this raised a question as to whether or not the treatment comparison in sheep was valid. To examine this possibility, we compared the difference in treatment means observed for animals in sequence 1 (Ampi-kel 10^R, Polyflex^R) and sequence 2 (Polyflex^R, Ampi-kel 10^R). These results for *AUC* and CMAX are provided in Figs 3 and 4.

[2] The test of the null hypothesis of no sequence effects was based upon the use of subjects nested within sequence as the appropriate error term.

Table 3. Confidence limits about the difference in treatment means for *AUC* and CMAX values

	Ratio	LCL	UCL	%CV ANOVA
Amp/Poly				
Calf *AUC*	0.87	0.82	0.89	5.1
Sheep *AUC*	0.81	0.79	0.83	3.5
Swine *AUC*	0.76	0.68	0.81	10.2
Calf CMAX	1.30	1.15	1.42	12.8
Sheep CMAX	1.12	0.95	1.22	13.8
Swine CMAX	1.12	0.89	1.22	19

With regard to *AUC*, treatment differences observed for animals in sequence 1 were markedly smaller than those observed for animals in sequence 2. For animals in sequence 1, the ratio of Ampi-kel 10^R/Polyflex^R was 1.04. For animals in sequence 2, this ratio was 0.64. Similar sequence-by-period interactions were not observed in the other two species.

With regard to CMAX (Fig. 4), the treatment differences associated with animals in sequence 1 were slightly greater than those of sequence 2. However, because of the lack of a concomitant period effect, this difference was rather small.

DISCUSSION

Interpretation of study results

The width and location of the confidence bounds were dissimilar across the three target animal species. Therefore, although statistically significant treatment-by-species interactions were not observed, differences in the conclusions regarding equivalence for the individual parameters did occur. This observation underscores a potential danger associated with the use of significance tests for rendering bioequivalence determinations. Tests of significance may fail to detect a clinically relevant difference between means when statistical power is low. Conversely, such tests may declare clinically unimportant differences to be significant when the statistical power is high.

The confidence interval approach to assessing product bioequivalence is based upon a range of acceptable differences. The location of the confidence bounds and the width of the confidence intervals are affected by three variables: subject number, the difference between treatment means (relative to the reference mean), and the unexplained noise in the dataset (expressed as the standard error of the estimate of these differences). In a two treatment, two period, two sequence crossover study, this unexplained noise includes within-subject variability.

To estimate the magnitude of the within-subject variability, an investigator must employ repeated measures designs whereby treatments are replicated within subjects. For drugs with a

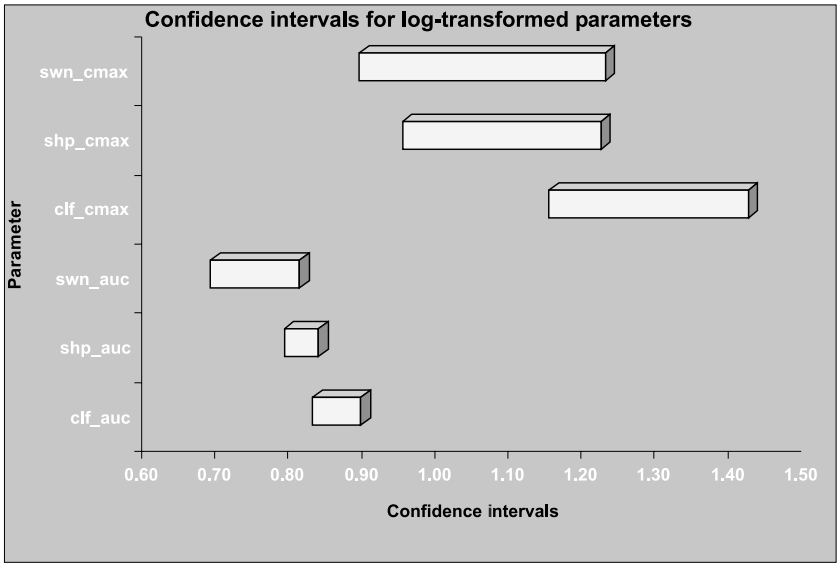

Fig. 2. Confidence intervals about the treatment difference for each ln-transformed parameter. All confidence intervals are expressed relative to the reference mean. swn_cmax = confidence interval associated with the treatment comparison of Ln-CMAX values in swine. shp_cmax = confidence interval associated with the treatment comparison of Ln-CMAX values in sheep. clf_cmax = confidence interval associated with the treatment comparison of Ln-CMAX values in calves. swn_auc = confidence interval associated with the treatment comparison of Ln – *AUC* values in swine shp_auc = confidence interval associated with the treatment comparison of Ln – *AUC* values in swine clf_auc = confidence interval associated with the treatment comparison of Ln – *AUC* values in swine.

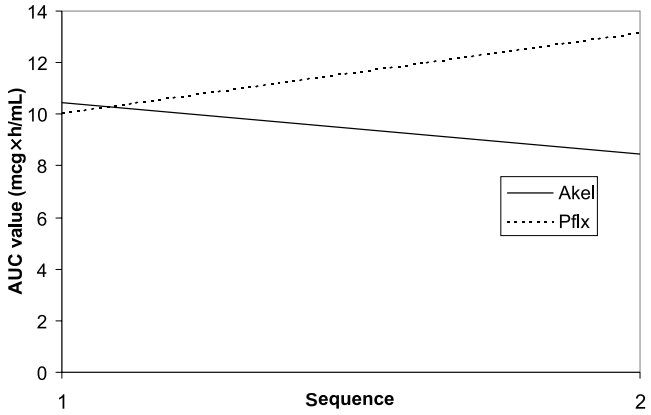

Fig. 3. Plot of the period-by-treatment interaction observed in the treatment comparison of *AUC* values in sheep.

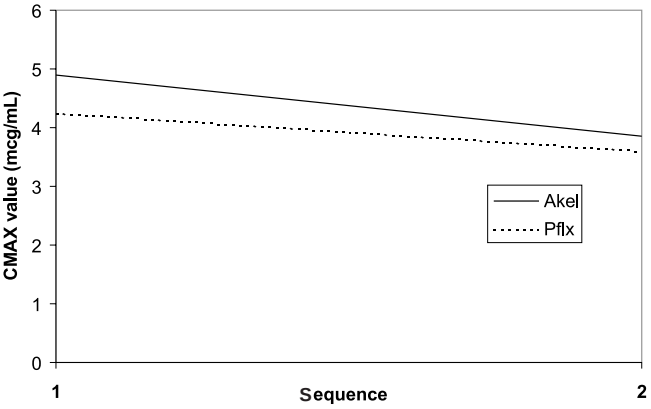

Fig. 4. Plot of the period-by-treatment interaction observed in the treatment comparison of CMAX values in sheep.

narrow therapeutic window, the use of extended crossover trials can ensure that the test and reference products are comparable in terms of within-subject profile repeatability. Several extended crossover designs and computational algorithms have been proposed by the Center for Drug Evaluation and Research (Anon 4). Depending upon the algorithm employed, extended crossovers can be used to assess the within-subject variability associated with the test and reference product, to identify potential subject-by-formulation interactions, and to adjust the bioequivalence confidence interval criteria based upon the within-subject variability associated with the reference product. In contrast, the Center for Veterinary Medicine does not currently recommend use of these complex study designs in veterinary species because of potential physiological changes that can bias study results (induced by growth or stress), an increased risk of animal dropout, and recognized economic constraints.

One of the difficulties that can occur when using a two treatment, two period, two sequence crossover design is that residual (carryover) effects from period 1 may influence the outcome of period 2. This phenomenon may simply appear as a statistically significant period effect if the magnitude of the carryover from treatment A equals that from treatment B. Period effects alone do not detract from the legitimacy of the treatment comparison. However, if the magnitude of the carryover effect differs between the two treatment groups (i.e. statistically significant period effects plus sequence effects), the resulting treatment comparison is considered to be invalid (Jones & Kenward, 1989). In that case, only period 1 data should be used in the assessment of product bioequivalence.

Unlike that observed in calves and swine, statistically significant sequence effects ($P < 0.05$) were observed in sheep (both *AUC* and CMAX). Moreover, in sheep, *AUC* values estimated from the period 1 dataset exceeded that of period 2. The order of treatment administration most greatly influenced ampicillin bioavailability associated with the Polyflex^R formulation. The within-treatment ratio of *AUC* values, periods 2 vs. 1 were 0.81 and 0.76 for Ampi-kel 10^R and Polyflex^R, respectively.

These results suggest that in sheep, prior exposure to ampicillin decreased the bioavailability of subsequent ampicillin administration when treatments were sequentially injected into a single site. The extent to which this apparent interference was attributable to a local response to the drug or to the excipients in the respective formulations is unclear. However, the presence of concomitant period and sequence effects does suggest that formulation influenced the magnitude of this interference. One explanation for the apparent unequal carryover includes the possibility that injection site residues of the Ampi-kel 10^R vehicle interfered with subsequent ampicillin absorption from the aqueous Polyflex^R suspension.

In general, formulation tended to have the least impact on the extent of ampicillin absorption in calves and the largest impact on the extent of absorption in swine. Conversely, formulation had a far greater effect on the CMAX values of calves as compared with that of the other two species. In fact, for CMAX, the confidence bounds collected in swine and sheep were nearly superimposable, both meeting the bioequivalence criteria of 0.80–1.25. This was not the case in calves. Thus, the results of the bioequivalence assessment in one target animal species could not be extrapolated to the other species. In this regard, it is interesting to note that in a recent publication, two subcutaneous ivermectin formulations differed in the rate of drug absorption in swine (seen as a shift in TMAX), but were virtually identical in cattle (Lifschitz *et al*., 1999). Therefore, species-by-formulation effects for parenteral dosage forms are clearly a possibility that should not be ignored.

Possible reasons for interspecies differences in parenteral product relative bioavailability

Potential factors that could affect interspecies differences in parenteral product relative bioavailability (other than intrasubject variability) include:

- Bias in product *AUC* estimates introduced by poor study design.
- The potential impact of elimination-rate constant on observed differences in product CMAX values.
- Interactions between animal physiology and product formulation.
- Species differences in what constitutes the rate-limiting step in drug absorption.
- The exaggeration of product inequivalence because of interspecies differences in label instructions for product use.
- These points are discussed below.

Bias in product AUC *estimates introduced by poor study design.* To explore the effect of blood sampling schedule on apparent differences in product bioavailability, we need to consider the relationship between drug pharmacokinetics, the time interval covered by the *AUC* value (i.e. partial *AUC* values), and the estimates of product relative bioavailability.

When a drug product demonstrates first order absorption and elimination, there should be little change in the test/reference *AUC* ratio once drug absorption is complete. Accordingly, confidence limits estimated shortly after TMAX should be nearly identical to those collected when *AUC* is extrapolated to time infinity (Lovering *et al*., 1975; Martinez & Jackson, 1991). In fact, for drugs associated with a long elimination half-life and highly variable $CL_{systemic}$, use of truncated *AUC* values may actually improve the ability to compare product extents of absorption (Endrenyi & Tothfalusi, 1997). However, the time associated with the quantifiable blood concentration could affect product *AUC* ratios when one or both formulations exhibit flip-flop kinetics (i.e. formulations where the rate of elimination is controlled by the rate of drug absorption) (Byron & Notari, 1976).

Although the terminal elimination half-life of i.m. Ampi-kel 10^R and Polyflex^R could not be determined in the current investigation, we know that quantifiable ampicillin blood concentrations extended up to and beyond 12 h postdose in all three target animal species. As more than 97% of an administered dose is eliminated within five elimination half-lives (assuming linear elimination kinetics), we estimate that the terminal elimination half-lives associated with i.m. administration of both Ampi-kel 10^R and Polyflex^R were at least 2.4 h (i.e. 12 h/5). In contrast, the terminal elimination half-life reported after intravenous (i.v.) ampicillin administration in sheep was been estimated to be between 0.32 and 1.12 h (Montesissa *et al*., 1994; Escudero *et al*., 1999). Calves tend to have the longest terminal elimination half-life, demonstrating average elimination half-life estimates of approximately 1.5 h (Montesissa *et al*., 1994). A report of i.v. ampicillin elimination half-life in swine was not available. Therefore, after comparing the ampicillin depletion rates associated with i.v. vs. i.m. administration, we conclude that both Ampi-kel 10^R and Polyflex^R are likely to be associated with flip-flop kinetics.

In the presence of flip-flop kinetics, the duration of blood sampling can markedly affect the accuracy of an *AUC* comparison. To illustrate this point, we simulated two blood concentration/time profiles (labelled test and reference products) in four animal species: A, B, C, and D. The parameters used to generate these simulations are summarized in Table 4. In each case, the extent of absorption (F) was set as 1.0 (i.e. 100% bioavailability). Therefore, the ratio of product *AUC* values, if extended to time infinity (i.e. F/CL) would always equal 1. The models differed only with regard to K01 (the absorption rate constant) and K10 (the elimination rate constant from the central compartment). All other parameter values were constant. For each set of simulations, the partial *AUC* values were estimated from time zero to hour *i*, where $i = 1$–24 (AUC_{0-i}).

The interspecies difference in the test/reference *AUC* ratios are provided in Fig. 5. For species A and B, both formulations exhibited flip-flop kinetics. For species C, test and reference formulations followed conventional pharmacokinetics (i.e. the terminal elimination rate constant was controlled by K10, rather than K01). However, in species D, flip-flop kinetics was only associated with the test product.

Using these simulated conditions, the apparent interspecies differences in product relative bioavailability greatly depended

Table 4. Simulation parameters. Constants = V_c (650 mL), Dose (100 mg), K12 and K21 (1 h^{-1})

	Species A	Species B	Species C	Species D
$K01_{test}$ (h^{-1})	0.5	0.5	1	0.3
$K01_{ref}$ (h^{-1})	0.3	0.3	2	2
K10 (h^{-1})	0.7	1	0.5	1
Time to >97% absorption of the test product (h)	6.93	6.93	3.5	11.6
Time to >97% absorption of the reference product (h)	11.6	11.6	1.7	1.7
AUC_{test} (μg × h/mL) estimated to time infinity	213	148	294	150
AUC_{ref} (μg × h/mL) estimated to time infinity	215	150	284	133
Ratio AUC_{test}/AUC_{ref}	0.99	0.99	1.03	1.13
$AUC_{inf–test}$ (μg × h/mL)*	220	154	308	154
$AUC_{inf–ref}$ (μg × h/mL)*	220	154	308	154
Ratio $AUC_{inf–test}/AUC_{inf–ref}$	1	1	1	1
$TMAX_{test}$ (h) observation	2	1	1	1
$TMAX_{ref}$ (h) observation	3	2	1	1
$CMAX_{test}$ (g/mL)†	33.49	28.46	52.58	20.75
$CMAX_{ref}$ (μg/mL)†	25.31	20.74	69.96	59.68
Ratio	1.32	1.37	0.75	0.351

K01 = Absorption rate constant; K10 = elimination rate constant.
*$AUC_{inf} = D \times F/Cl_{systemic}$.
†Model-based CMAX value.

upon the time interval covered by the partial *AUC*. The impact of prolonged absorption was most apparent at the earliest sampling times where differences in product K01 values exerted its greatest effect. Not surprisingly, whenever flip-flop kinetics were present, test/reference $AUC_{0–i}$ ratios continued to change until nearly all of the drug molecules were absorbed.

As the test product was absorbed more rapidly than the reference in species A and B, the test/reference *AUC* ratios started well above unity and subsequently dropped towards 1. This would incorrectly lead to the conclusion that the bioavailability of the test product was substantially greater than that of the reference. It is important to note that as the formulation effects were simulated to be species-independent (i.e. K01 values for the test and reference product were identical in species A and B), interspecies differences in drug clearance did not affect the ratio of the partial *AUC* estimates. In contrast, when neither the test nor the reference product exhibited flip-flop kinetics (species C), the partial *AUC* ratios reached unity at about the time of TMAX. Therefore, without adequate consideration given to area covered by a partial *AUC* value, if the relative

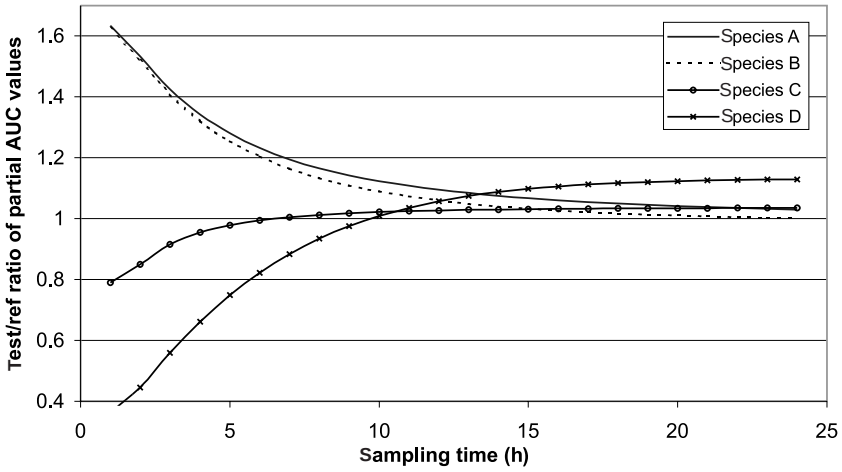

Fig. 5. Relationship between sampling time and test/reference ratio of partial *AUC* values as relationship between absorption and elimination rates are varied. In each case, both products are 100% bioavailable and therefore the true ratio of test/reference $AUC_{0–inf}$ values should equal 1. Deviations from expected ratios attributable to bias introduced by sampling times (refer to text). Simulations are based upon a two-compartment open body model where $V_c = 650$ mL, dose = 100 mg and where both K12 and K21 = 1 h^{-1}. The absorption and elimination rate constants for each species (expressed as h^{-1}) are as follow: Species A: K10 = 0.7, K01 (test) = 0.5, K01 (ref) = 0.3 (ratio of K01 test/K01 ref = 1.67) FLIP-FLOP kinetics observed with both test and reference products. Species B: K10 = 1.0, K01 (test) = 0.5, K01 (ref) = 0.3 (ratio of K01 test/K01 ref = 1.67) : FLIP-FLOP kinetics observed with both test and reference products. Species C: K10 = 0.5, K01 (test) = 1.0, K01 (ref) = 2.0 (ratio of K01 test/K01 ref = 0.5) – no flip flop. Species D: K10 = 1.0, K01 (test) = 0.3, K01 (ref) = 2.0 (ratio of K01 test/K01 ref = 0.15) : FLIP-FLOP observed with test product only.

bioavailability of species C were compared with that of either species A or B, we might incorrectly conclude that the extent of product bioavailability was markedly affected by a species-by-formulation interaction.

The profile in species D provided an opportunity to demonstrate an additional potential source of error. Incorrect conclusions regarding product relative bioavailability can occur if the sampling times fail to adequately capture CMAX. In this example, the initial blood sample occurred after the true TMAX of the reference product (0.56 h based on the pharmacokinetic model), resulting in an 8.5% bias in CMAX values (51.24 μg/mL vs. 59.68 μg/mL for observed and actual values, respectively). The resulting loss in area enabled the test product to appear to be more bioavailable than the reference. As seen by the inconsistency between AUC_{test}/AUC_{ref} (ratio of observed *AUC* values extrapolated to time infinity) vs. $AUC_{inf-test}/AUC_{inf-ref}$ (model-based *AUC* values) this error could not have been predicted simply by extrapolating *AUC* values to time infinity (Table 4).

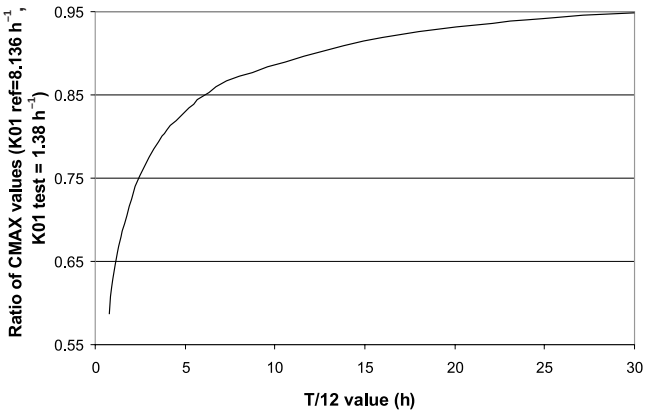

Fig. 6. Effect of varying the elimination half-life on test/reference CMAX ratios. In this example, the absorption rate constant of the test and reference product are fixed at 1.38 and 8.136 h^{-1}, respectively. Test/reference CMAX ratios (*Y*-axis) are plotted as a function of the drug's terminal elimination half-life (*X*-axis).

Relationship between K01 and K10: its impact on product bioequivalence. The next consideration is whether or not species differences in $CL_{systemic}$ can affect the interspecies comparison of test/reference CMAX values. Evidence that this could occur is seen by the differences in product CMAX ratios across species A vs. B (Table 4). In that example, although the test and reference K01 values were 0.5 h^{-1} and 0.3 h^{-1}, respectively, in both species, the corresponding CMAX ratios were 1.32 and 1.37. The only parameter that differed between these two datasets was the elimination rate constant, K10.

To explore the relationship between CMAX, K01 and K10, we evaluated the interaction between CMAX, K01 and K10. To accomplish this objective, we determined the change in CMAX ratios as we varied the ratio of $K01_{test}/K01_{ref}$. For simplicity, simulations were based on the following one-compartment open body model equation:

$$\text{CMAX} = \frac{\text{K01} \times FD}{V\,(\text{K01 - K01})} \times (\exp(-\text{K01} \times \text{TMAX} - \exp(-\text{K01} \times \text{TMAX})$$

$$\text{TMAX} = \frac{1}{\text{K01} - \text{K10}} \times \ln\,(\text{K01} / \text{K10})$$

where *V*, *D* and *F* were constant.

As expected, for any value of K10, the ratio of $CMAX_{test}/CMAX_{ref}$ approached unity as the test and reference K01 values converged (Fig. 6). Moreover, for any ratio of K01 values, the test/reference CMAX ratio moved closer toward unity as K10 decreased. Thus, the larger the differences between K01 and K10, the smaller the impact of interproduct differences in K01. In other words, if two products are associated with inequivalent rates of drug absorption, this difference will have less impact on the CMAX comparison in a species demonstrating a long elimination half-life as compared with one with a short elimination half-life. This interdependency is further illustrated in Fig. 7 where the variation in test/ref CMAX ratios (for several sets of test/ref K01 ratios) is plotted as a function of terminal elimination half-life (where half-life equals 0.693/K10 for a one compartment model).

Thus, one prerequisite for extrapolating product relative bioavailability across target animal species should be that these species exhibit comparable elimination half-lives. Otherwise, even if the differences in product *in-vivo* dissolution are identical across species, the resulting comparison of CMAX values could be markedly different.

True interspecies differences in the rate and/or extent of drug absorption. As demonstrated in this investigation, we cannot simply assume that the relative bioavailability of two i.m. or s.c. dosage forms will be comparable across animal species. In addition to differences in $CL_{systemic}$, other cues for avoiding interspecies extrapolations of parenteral dosage forms include the presence of active drug metabolites (because of potential species-related differences in drug metabolism), the possibility of nonlinear pharmacokinetics, and the suspicion that there may be species-specific sensitivity to product excipients. Nevertheless, even when these variables appear to be of no concern, there continues to exist an uncertainty as to whether or not there may be true species-related differences in formulation effects.

Within this investigation, evidence strongly suggests that product formulation did not have comparable effects on ampicillin absorption characteristics across the three target animal species. This leads us to the question of whether or not it would be possible to identify a category of compounds or formulations for which it would be safe to extrapolate parenteral product bioequivalence across animal species. Amidon *et al.* (1995) noted that orally administered drugs could be segregated into one of four categories based upon their GI permeability and aqueous solubility. This work became the basis for the biopharmaceutics classification system (BCS).

The BCS is used to determine the types of studies needed to confirm product bioequivalence (Anon 2 and 3). It is based upon the categorization of drugs into one of four possible classes:

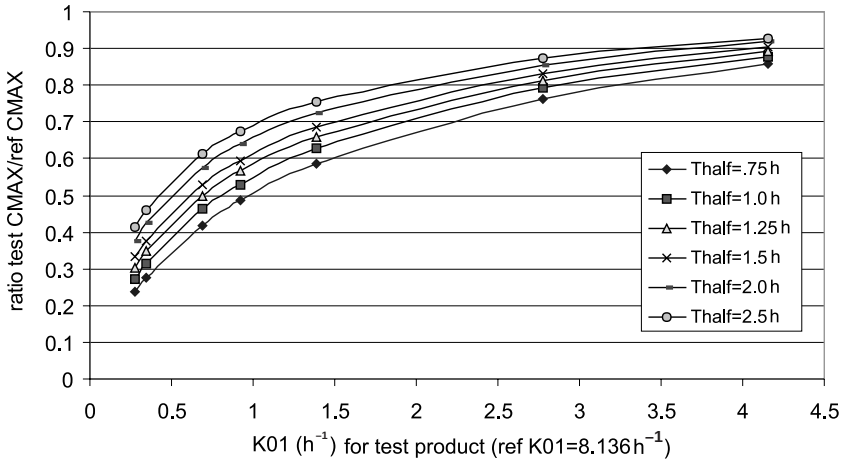

Fig. 7. Effect of elimination half-life on the ratio of product CMAX values. For any given elimination half-life, the absorption rate constant for the test product was varied. In each case, the absorption rate constant of the reference product was held constant at 8.136 h^{-1}. Within each half-life estimate, the resulting ratio of test/reference CMAX value is plotted as a function of the test product absorption rate constant.

- *Class I*: high aqueous solubility, high gastrointestinal (GI) permeability, rapid dissolution. For these compounds, the critical process is gastric emptying. Formulation changes can be accepted on the basis of a single-point *in-vitro* dissolution test if conducted in several media.
- *Class II*: low aqueous solubility, high GI permeability. The critical process for these compounds is *in-vivo* dissolution. For formulation changes, *in-vivo* bioequivalence studies are generally required. Alternatively, bioequivalence can be confirmed on the basis of multiple dissolution profiles if an *in-vivo*/*in-vitro* correlation has been established.
- *Class III*: High solubility, low permeability. For these compounds, absorption is permeability-rate limited. Therefore, it is highly unlikely that an *in-vivo*/*in-vitro* correlation can be established.
- *Class IV*: Low solubility, low permeability. For these complex drugs, *in-vivo* bioequivalence data should be required.

Unfortunately, similar work has not be applied to parenteral dosage forms. While we may be able to extract some information from CDERs BCS, it is critical that the differences between the absorption kinetics of a parenteral (i.m. and s.c.) vs. oral formulations be recognized. Accordingly, if the BCS is to be applied to parenteral dosage forms, some of the critical variables need to be redefined. For example, while it may be inappropriate to apply oral permeability data to predict the tissue permeability of parenteral dosage forms, we may still be able to classify compounds as highly permeable if the i.m. or s.c. product demonstrates an absolute bioavailability exceeding 90%. Conversely, what constitutes a highly soluble drug may need to be redefined as the fluid volume associated with a parenteral injection is far less than that encountered in the gastrointestinal tract.

Numerous biological factors can influence the absorption of a drug from an injection site (MacDiarmid (1983). These include:

- The area of the absorptive surfaces in contact with the injection volume.
  1. Ability of the injected volume to diffuse into surrounding tissues.
  2. Rate at which the solvent is removed. The rate of diffusion of dissolved drug through the connective tissue substance determines the nature of the resulting concentration gradient of solute molecules. Very high drug concentrations near the undissolved solute particle can affect the rate of drug dissolution.
- Blood and lymph flow.
  1. Exercise-induced modification of tissue blood flow, resulting in enhanced drug absorption.
  2. Anatomical differences in degree of vascularization.
- Permeability of blood and lymph vessels. This can be altered by some excipients (direct affect and inflammation) and by trauma caused by injection.
- Tissue dissolution conditions such as local pH, temperature, and movement.
- Binding of drug or product excipients to tissue constituents.
- If a drug is formulated in an oil vehicle, the partition coefficient of the drug between tissue fluids and vehicle will govern the rate at which it is absorbed.
- Ability of tissue to respond to changes in osmotic pressure whereby increasing the osmotic pressure of a formulation can result in increased fluid volume in the injection site.
  1. Increases in fluid volume can decrease the rate of drug absorption for dissolved drug (permeability rate limited absorptive process).
  2. Increases in the concentration of a suspension can delay drug dissolution and decrease the rate of drug absorption (dissolution-rate limited absorptive processes).
- Ability of drug to diffuse through the formulation and tissue fluids.
- Local drug metabolism.

Within this list, there exist a wide variety of physiological variations between species that could account for differences in product relative bioavailability (MacDiarmid, 1983; Pope, 1984). This includes dissimilarity in blood supply, ability of drug and/or vehicle to diffuse through the ground substance, composition of tissue fluids, capillary permeability, local response to drug or product excipients, volume of tissue fluid, response to changes in osmotic pressure, and surface area available for drug/tissue contact (Baggot & Brown, 1998). In this study, we observed a consequence of this diversity when unequal carry-over effects were observed in sheep. While we do not know the

cause of this effect, it does demonstrate that diversity in the physico-chemical properties of muscle tissues can potentially affect drug stability and tissue–solvent interaction. Furthermore, simply because a product is formulated as a true aqueous solution does not necessarily imply that bioequivalence problems will not exist. Solubilized drug contained with an i.m. or s.c. injection can re-precipitate at the site of injection. This may be because of preferential absorption of the vehicle by the surrounding tissues or by the effect of local pH on drug ionic form.

Another possible source of interspecies inconsistencies could be differences in the rate-limiting step for product absorption. As described for oral dosage forms, the effect of varying the rate of *in-vivo* dissolution on *in-vivo* product bioequivalence depends upon whether or not drug dissolution is the rate limiting step in the absorption process (Kaus *et al*., 1999). As there may be interspecies differences in tissue permeability and diffusion capacity, there may likewise be inconsistencies in the relationship between *in-vivo* dissolution and K01 values.

Finally, although absorption kinetics is most often represented as a first order function, complex kinetics absorption may be present (i.e. where the absorption rate is best described by a polyexponential function). In fact, drug absorption from i.m. sites rarely follow first-order kinetics as factors governing absorption (such as the concentration gradient and surface area) often change as the drug is absorbed (Baggot & Brown, 1998). As multifunctional absorption kinetics usually reflect complex interactions between animal physiology and formulation chemistry, interspecies extrapolations should not be considered in instances where this is likely to occur (e.g. compounds associated with high permeability but low aqueous solubility). Unfortunately, as demonstrated in the simulated example by Koritz (1994), the presence of complex absorption processes may not always be apparent.

Interspecies differences in label instructions. Interspecies differences in the relative bioavailability of two formulations may also result from differences in the use instructions described on the approved product label. For example, larger animals may require larger injection volumes. Injection volume, in and of itself, can affect the rate and extent of drug absorption. Therefore, even in the case of readily dissolved drugs or drugs in solution, the increase in injection volume can result in slower drug absorption (MacDiarmid, 1983). It should be noted that in the current investigation, different injection volumes were necessitated by differences in the label doses for ampicillin trihydrate in swine and cattle.

The anatomical site of injection may also differ across species. As anatomical regions can significantly alter the rate and extent of drug absorption within a given species (Marshall & Palmer, 1980; Rutgers *et al*., 1980; Firthe *et al*., 1986), it would not be surprising if the physiological differences in anatomical regions also affect product relative bioavailability. We must also ask if differences in product relative bioavailability may result when the required dosage results in the need for same muscle, multiple injections in one species but not in another.

Concluding remarks

There exist numerous physiological and physico-chemical factors that can affect our ability to extrapolate product bioequivalence across target animal species. For some of these variables, criteria for interspecies extrapolations are becoming increasingly evident. For example, bioequivalence extrapolations should not occur between species with markedly different elimination kinetics. However, for others, the complexities of these interactions obscure our ability to identify and categorize these variables.

This void in our understanding raises the question of whether or not we have the knowledge required to enable the accurate prediction of product behavior under various disease states. Particularly with the burgeoning growth of companion animal medicine, we are approaching a rise in problems that mimic those encountered in human medicine. Are we certain that products found to be equivalent in young, healthy animals will also be equivalent in geriatric or pediatric populations? Will the product found to be bioequivalent in normal healthy animals continue to be bioequivalent in diseased animals? In this regard, we already know that changes in animal physiology can significantly affect drug pharmacokinetics (Martinez & Berson, 1998). As seen with the blood concentration profiles resulting from i.m. injection of amoxicillin aqueous suspension in calves ranging in weight from 45 to 62 kg (dosed at 7 mg/kg), both age and weight can also markedly affect the rate and extent of drug bioavailability (Marshall & Palmer, 1980). Clearly, more research is needed in to address this issue.

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