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Pharmacokinetics, pulmonary disposition and tolerability of liposomal gentamicin and free gentamicin in foals

A. J. BURTON, S. GIGUÈRE* and R. D. ARNOLD†

Department of Large Animal Medicine, College of Veterinary Medicine, University of Georgia, Athens, USA
†Department of Drug Discovery and Development, Harrison School of Pharmacy, Auburn University, Alabama, USA.

*Correspondence email: gigueres@uga.edu; Received: 03.03.14; Accepted: 12.06.14

Summary

Reasons for performing the study: Although gentamicin is highly active against *Rhodococcus equi in vitro*, its clinical efficacy has been limited presumably due to poor cellular uptake. Encapsulation of drugs in liposomes enhances their cellular uptake.
Objectives: To compare the disposition of liposomal gentamicin (LG) and free gentamicin (FG) in the plasma, pulmonary epithelial lining fluid and bronchoalveolar cells of healthy foals after i.v. administration or by nebulisation, and to assess the tolerability of the drug after repeated i.v. dosing.
Study design: Experimental study.
Methods: Eight healthy foals received a single i.v. or nebulised dose (6.6 mg/kg bwt) of LG or FG in a balanced Latin square design, with a 14-day washout period between treatments. Subsequently, 12 healthy foals were given either LG or FG at 6.6 mg/kg bwt i.v. q. 24 h for 7 doses and urinary protein, creatinine, γ-glutamyltransferase and electrolytes were measured on Days 0, 3 and 7 to quantify renal injury. Concentrations of gentamicin were measured using liquid chromatography-tandem mass spectrometry.
Results: After i.v. administration, LG had a significantly higher mean (± s.d.) half-life (16.3 ± 3.5 vs. 6.2 ± 1.8 h) and volume of distribution (2.00 ± 1.03 vs. 0.72 ± 0.32 l/kg bwt) compared with FG. Peak gentamicin concentrations in bronchoalveolar cells were significantly higher for LG compared with FG after administration by both the i.v. (5.27 ± 2.67 vs. 2.98 ± 1.67 mg/l) and the nebulised (4.47 ± 2.66 vs. 1.49 ± 0.57 mg/l) routes. Liposomal gentamicin was well tolerated by all foals and indices of renal injury were not significantly different from those of foals administered FG.
Conclusions: Administration of LG is well tolerated and results in higher intracellular drug concentrations than FG. Liposomal gentamicin warrants further investigation for the treatment of infections caused by intracellular pathogens such as *Rhodococcus equi*.

Keywords: horse; gentamicin sulfate; aminoglycoside; foal; pharmacokinetics; liposomes

Introduction

Rhodococcus equi, a Gram-positive facultative intracellular bacterium, is a common cause of pneumonia in foals aged 1–5 months. Despite recommended therapy with the combination of a macrolide and rifampin, the mortality rate of severely affected foals is still around 30% [1,2]. Over the last 10 years, the incidence of macrolide and rifampin resistance has increased [3] and resistant isolates of *R. equi* are cultured from up to 40% of affected foals at some farms [4]. All *R. equi* isolates from pneumonic foals, including macrolide-resistant isolates, are susceptible to the aminoglycoside gentamicin *in vitro* [3,5]. Additionally, gentamicin is one of the few antimicrobial agents that are bactericidal against *R. equi* [6,7]. Although gentamicin is highly active against *R. equi in vitro*, its efficacy *in vivo* has been limited [8] presumably because of poor cellular uptake. A delivery system that could improve intracellular concentrations of gentamicin would be likely to increase its *in vivo* efficacy against *R. equi*.

Encapsulation in liposomes is one method by which the intracellular penetration of drugs might be enhanced. Liposomes are lipid vesicles 0.08–5 μm in diameter composed of one or several amphiphile bilayers surrounding an aqueous core [9,10]. After administration, liposomes are phagocytised and rapidly localise in the phagosomes of mononuclear cells [11]. They also extravasate at sites of increased vascular permeability and are readily taken up into inflamed tissues, enabling targeted delivery to infected organs [9,12]. Previous studies in a variety of species have shown that, compared with free (conventional) gentamicin (FG), liposomal gentamicin (LG) has significantly enhanced cellular penetration and activity against facultative intracellular bacteria such as *Listeria monocytogenes, Mycobacterium avium, Salmonella* spp. and *Brucella abortus* both *in vitro* and *in vivo* [10,12–16]. Recently, the safety and biodistribution of i.v. ^{99m}Tc-25 labelled liposomes was examined in mature horses [17]. Liposomes distributed primarily to the lungs, liver, spleen and kidneys and no adverse effects were noted [17]. In a soft tissue abscess model in horses, liposomes concentrated and persisted at the site of infection [18].

We hypothesised that administration of LG to foals is well tolerated after i.v. administration or nebulisation and results in increased concentrations of the drug in bronchoalveolar (BAL) cells relative to FG. The objectives of this study were to compare the disposition of LG and FG in the plasma, pulmonary epithelial lining fluid (PELF) and BAL cells of healthy foals after administration by the i.v. route or by nebulisation, and to assess the tolerability and accumulation of LG vs. FG after repeated i.v. dosing.

Materials and methods

Formulation of LG

Liposomal gentamicin was formulated by aqueous capture using 1,2-dipalmitoyl-sn-glycero-3-phosphocholine[a], cholesterol[b] and 1,2-distearol-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000][a] in chloroform in a molar ratio of 9:5:1, which were stored in chloroform at -80°C, then thawed and mixed in a 500 ml glass round bottom flask. The chloroform was evaporated from the mixture using vacuum under a constant nitrogen stream (Rotavapor R-210/R-215)[c] and the resultant thin lipid film was rehydrated with aqueous gentamicin sulfate[d] (2500 mg/l) at a ratio of 40.5 mg/μmol of lipid (resultant lipid concentration of 5 mmol/l). After 5 freeze–thaw cycles in liquid nitrogen, the particles were sized using 3 passes through a high-pressure homogeniser (EmulsiFlex-C5)[e]. Nonencapsulated gentamicin was removed via 3 rounds of dialysis (Slide-A-Lyzer dialysis cassettes, 10K MWCO)[f] in 0.9% saline at 4°C. Mean percentage of initial gentamicin remaining encapsulated was 24.9% (range 20.1–32 %). Final particle size was verified using a dynamic light scattering particle sizer (90Plus Particle Size Analyzer)[g]. Median particle size diameter was 158 nm (range 139–178 nm) and mean (± s.d.) polydispersity index was 0.114 ± 0.020. Liposomal gentamicin was stored in the dark at 4°C and administered within 3 weeks of formulation.

Animals

Twenty Quarter Horse foals weighing 80–204 kg depending on age were used. Foals were considered healthy on the basis of physical examination, complete blood count and plasma biochemical profile. The foals were kept with their dams in individual stalls during the experiments and on pasture between experiments with *ad libitum* access to grass hay and water.

Experimental design and sample collection

Study 1*: Single dose i.v. or nebulised LG or FG:* Eight foals received a single i.v. or nebulised dose (6.6 mg/kg bwt) of FG[h] or LG in a balanced Latin square design. Beginning at age 5–7 weeks, every foal received each of the 4 possible drug-route combinations with a 14-day washout period between each administration. Intravenous FG and LG were diluted in 250 ml of sterile 0.9% saline and administered via a jugular catheter as a constant rate infusion over 15 min. Nebulised FG and LG were administered by inhalation over 15 min via a commercial equine nebuliser (Flexineb)[i]. To ensure equivalent rate of delivery, FG was diluted to the same volume as LG with sterile 0.9% saline. Particle size of nebulised LG was verified using laser diffraction (Spraytec)[j]. Blood samples for plasma separation were obtained from a catheter placed in the contralateral jugular vein prior to each drug administration, and at 5, 10, 20, 30 and 45 min, and 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24 and 48 h after the end of the 15 min i.v. infusion or nebulisation period. Bronchoalveolar fluid was collected at 2, 4, 8, 24 and 48 h. Foals were sedated with xylazine hydrochloride (0.5 mg/kg bwt i.v.) and butorphanol tartrate (0.07 mg/kg bwt i.v.) prior to collection of BAL fluid. Prior to analysis for gentamicin concentration, blood samples were centrifuged at 400 × ***g*** for 10 min and the resultant plasma frozen at -80°C until assayed.

Study 2*: Repeated dose i.v. LG or FG:* Twelve foals aged 5–7 weeks were given either LG (6 foals) or FG (6 foals) at 6.6 mg/kg bwt i.v. q. 24 h for 7 doses. Each dose was diluted in 250 ml of sterile 0.9% saline and administered over 15 min via an indwelling 14 gauge catheter placed in a jugular vein. Blood samples for plasma separation and measurement of gentamicin concentrations were obtained on Day 1 and on Day 7 at the times listed for *Study 1*. Bronchoalveolar fluid was collected 2, 6 and 24 h after the end of infusion on Day 7. Urine was collected the day prior to the first drug dose (Day 0) and again 2 h after the end of the infusion on Days 3 and 7. Foals were sedated with xylazine hydrochloride (1.1 mg/kg bwt i.v.) and anaesthetised with ketamine (2.2 mg/kg bwt i.v.) to allow passage of a urinary catheter using aseptic techniques. Urinalysis was performed and urine and concurrently obtained plasma samples were submitted for measurement of creatinine, γ-glutamyltransferase (GGT), protein, calcium, chloride, magnesium, sodium and potassium concentrations. Fractional excretion of electrolytes, urinary GGT to creatinine ratio and urinary protein to creatinine ratios were calculated. Prior to analysis for gentamicin concentration by liquid chromatography tandem mass spectrometry (LC-MS/MS), urine and plasma samples were centrifuged at 400 × ***g*** for 10 min and the supernatant was frozen at -80°C until assayed.

Bronchoalveolar lavage and processing

A 10 mm diameter, 2.4 m BAL catheter[k] was passed via nasal approach until wedged into a bronchus. The lavage solution consisted of 4 aliquots of 60 ml 0.9% saline solution infused and instantly aspirated. Immediately upon collection, the total volume of BAL fluid recovered was measured and a 3 ml aliquot saved in an EDTA tube from which total nucleated cell count was determined by use of a cell counter (Cellometer Auto T4)[l]. Bronchoalveolar fluid was immediately centrifuged at 400 × ***g*** for 10 min. The BAL cells in the resultant pellet were washed, re-suspended in 500 μl of acetonitrile: 0.2% formic acid (1:1, *v/v*), vortexed and frozen at -80°C until assayed. Supernatant BAL fluid was also frozen at -80°C until assayed. Before assaying, the cell pellet samples were thawed, vortexed vigorously and sonicated for 10 min to ensure complete cell lysis. The resulting suspension was centrifuged at 500 × ***g*** for 10 min and the supernatant fluid was used to determine the intracellular concentrations of gentamicin.

Drug analysis in plasma and body fluids by LC-MS/MS

The concentration of gentamicin sulfate was measured using LC-MS/MS as previously described [19]. Briefly, gentamicin was extracted from plasma (250 μl) and urine (500 μl) using protein precipitation with an equal volume of ice-cold acetonitrile: 0.2% formic acid (9:1, *v/v*). Extracted samples were centrifuged (2°C at 10,000 × ***g*** for 10 min) twice. A 200 μl sample of each supernatant was transferred to polypropylene inserts for injection. The supernatant derived from the lyzed BAL cell pellet was transferred to low-volume polypropylene inserts for direct injection without further processing. To measure gentamicin concentration in PELF, 20 ml of the initial BAL fluid supernatant was thawed, acidified with formic acid (99.9%) and centrifuged at 1500 × ***g*** for 10 min. An aliquot of the resultant supernatant was mixed with an equal volume of ice-cold acetonitrile, centrifuged (2°C at 10,000 × ***g*** for 10 min) and 200 μl was transferred to polypropylene insert for injection. Calibration standards were prepared in drug-free foal plasma, BAL fluid or urine and then extracted as described above so that standard curves specific to each biologic matrix could be constructed. Concentration ranges of gentamicin sulfate used to construct standard curves and lower limits of quantification (LOQ) were: plasma 0.045–100 mg/l (LOQ, 0.045 mg/l), urine 3.125–100 mg/l (LOQ, 3.125 mg/l) and BAL fluid 0.001–6.25 mg/l (LOQ, 0.001 mg/l). The interassay coefficient of variation was <10% at concentrations 100–6.25 mg/l and ≤20% at concentrations <6.25 mg/l. Analyte separation and LC-MS/MS measurement of gentamicin were performed exactly as described previously [19] except that BAL fluid samples were introduced in the MS with a flow rate of 0.18 ml/min and a total run time of 8 min.

Calculation of gentamicin concentrations in PELF and BAL cells

Estimation of the volume of PELF was determined by the urea dilution method [20]. Urea nitrogen concentrations in BAL fluid ($Urea_{BAL}$) and concurrent plasma samples ($Urea_{PLASMA}$) were determined by use of a commercial quantitative colorimetric kit (Urea Assay Kit).[m] The volume of PELF (V_{PELF}) in BAL fluid was derived from the following equation: $V_{PELF} = V_{BAL} \times (Urea_{BAL}/Urea_{PLASMA})$, where V_{BAL} is the volume of recovered BAL fluid. The concentration of gentamicin in PELF (Gm_{PELF}) was derived from the following relationship: $Gm_{PELF} = Gm_{BAL} \times (V_{BAL}/V_{PELF})$, where Gm_{BAL} is the measured concentration of gentamicin in BAL fluid supernatant. The concentration of gentamicin in BAL cells (Gm_{CELLS}) was calculated using the following relationship: $Gm_{CELL} = (Gm_{PELLET}/V_{CELL})$ where Gm_{PELLET} is the measured concentration of gentamicin in the cell pellet supernatant and V_{CELL} is the mean volume of BAL cells. A volume of 1.20 μl per 10^6 BAL cells was used for calculations based on previous studies in foals [21].

Pharmacokinetic analysis

For each foal, plasma, PELF and BAL cell concentration vs. time data were analysed using commercial software (PK Solutions 2.0)[n]. Noncompartmental analysis was used for PELF and BAL cell data. A conventional linear 2-compartment model with weighting by the inverse of the model (1/y) best predicted i.v. plasma gentamicin data based upon computer assisted examination of residual plots, goodness of fit and the sum of squares. The equation $C_t = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t}$ was used, where C_t is the serum drug concentration at time t; *e* is the base of the Naperian logarithm; A and α are the intercept and rate constant, respectively, of the distribution phase; B and β are the intercept and rate constant, respectively, of the elimination phase. The rate constant of the elimination phase (β) was determined by linear regression of the terminal phase of the logarithmic plasma concentration vs. time curve using a minimum of 3 data points. Terminal half-life ($t_{½\beta}$) was calculated as 0.693/β. The area under the concentration-time curve (AUC) and the area under the first moment of the concentration-time curve (AUMC) were calculated using the trapezoidal rule, with extrapolation to infinity using C_{24h}/β, where C_{24h} is the plasma concentration at the 24 h sampling time. Mean residence time was calculated as: $AUMC_{0-\infty}/AUC_{0-\infty}$. Apparent volume of distribution based on the AUC was calculated as: $dose/AUC_{0-\infty} \cdot \beta$, apparent volume of distribution at steady state was calculated as: $dose \cdot AUMC_{0-\infty}/(AUC_{0-\infty})^2$, and systemic clearance was calculated from: $dose/AUC_{0-\infty}$.

Data analysis

Normality and equality of variance of the data were assessed with use of the Shapiro–Wilk and Levene tests, respectively. Data that were not normally distributed were log or rank transformed. For *Study 1*, the paired *t* test or the Wilcoxon rank sum test was used to compare i.v. pharmacokinetic variables between LF and FG. The effects of drug (LG vs. FG), administration route (i.v. vs. nebulised) and the interactions between drug and administration route on PELF and BAL cell pharmacokinetic

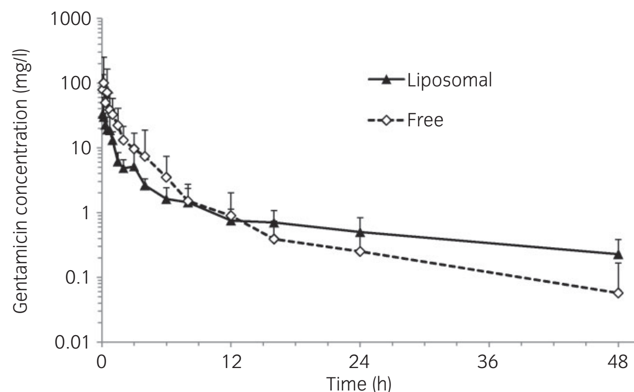

Fig 1: Mean (+ s.d.) plasma concentrations of gentamicin after i.v. administration of free gentamicin or liposomal gentamicin at a dosage of 6.6 mg/kg bwt to 8 foals (*Study 1*).

variables were assessed using a 2-way ANOVA for repeated measurement. For *Study 2*, the effects of drug (LG vs. FG), time (Day 0, Day 3 and Day 7), and the interactions between drug and time on renal indices were assessed using a 2-way ANOVA with one factor repetition (time). When warranted, multiple pairwise comparisons were done using the Holm–Sidak test. The paired *t* test or Wilcoxon rank sum test was used to compare i.v. pharmacokinetic variables obtained on Day 1 to those obtained on Day 7. Significance was set at $P<0.05$.

Results

Study 1: Single dose i.v. or nebulised LG vs. FG

Plasma concentration vs. time data after i.v. administration of FG and LG are presented in Fig 1. Intravenous administration of LG resulted in significantly lower initial plasma concentrations but significantly higher Vd, $t_{1/2\beta}$, mean residence time (MRT), and concentrations at 24 and 48 h compared with administration of FG (Table 1). The median particle size of nebulised LG was 3.1 μm with 71% of the particles being <5 μm and 92% being <10 μm. Plasma concentrations of gentamicin after administration of nebulised LG or FG were ≤0.78 mg/l at all time points.

Regardless of route of administration, gentamicin concentrations in BAL cells, time to maximum concentration (T_{max}), and AUC_{0-t} were significantly higher for LG than for FG (Table 2). Conversely, maximum concentration (C_{max}) in PELF was significantly higher after administration of FG compared with LG for both the i.v. and nebulised administration routes (Table 2). Similarly C_{max} in PELF was significantly higher after nebulisation than after i.v. administration regardless of drug (Table 2).

Study 2: Repeated dose i.v. LG or FG

Plasma pharmacokinetic variables obtained after administration of the first dose of LG or FG were not significantly different from those obtained in *Study 1* and not significantly different from those calculated after administration of the same formulation on Day 7 (data not shown), indicating no accumulation of either LG or FG in plasma over one week of daily administration. Daily i.v. administration of LG resulted in significantly higher C_{max} (12.1 ± 5.9 vs. 6.7 ± 1.9 mg/l; P = 0.015) and AUC_{0-t} (200 ± 82.9 vs. 105 ± 35.1 mg•h/l; P = 0.007) in BAL cells compared with FG. Concentration in BAL cells at 24 h (8.9 ± 7.2 vs. 3.5 ± 1.8 mg/l, P = 0.053) and T_{max} (median = 6 h, range = 2–24 h for both groups; P = 0.86) were not significantly different between LG and FG. There were no significant differences in gentamicin concentrations in urine between drug formulations or over time (Table 3). Indices of renal injury did not differ significantly between LG and FG. However, the mean fractional excretions of sodium and chloride were significantly greater on Day 7 compared with Day 0 or Day 3 for both LG and FG (Table 3). Urinary pH and GGT:creatinine ratio were significantly different between treatment groups on Day 0 (prior to drug administration). Therefore, these 2 parameters were expressed as a change from baseline (value on a given day – value on Day 0) for data analysis (Table 4). For both LG and FG, the difference in GGT:creatinine ratio was significantly higher on Day 7 compared with Day 3. The difference in urine pH was not significantly different between Day 3 and Day 7 but was significantly higher in foals that received LG compared with foals that received FG. One foal from each treatment group had casts on urine sediment analysis on Day 7. Three foals developed thrombophlebitis, 2 from the FG group and one from the LG group. One foal from each group developed mild self-limiting diarrhoea during treatment.

Discussion

In a prior study, age was found to have a profound effect on the pharmacokinetics of FG administered i.v. to foals [19]. The dose of 6.6 mg/kg bwt used in this study was based on simulations from data collected after administration of FG at a dose of 12 mg/kg bwt in the aforementioned study [19]. The mean (± s.d.) measured plasma concentration 1 h after i.v. administration of FG to foals aged 5–7 weeks in the present study (32.60 ± 25.28 mg/l) was similar to predicted concentrations (25.27 ± 9.52 mg/l at age 4 weeks and 34.52 ± 14.11 mg/l at age 12 weeks) [19]. Similarly, measured concentrations 24 h after administration in this study (0.25 ± 0.19 mg/l) compared closely with predicted concentrations (0.20 ± 0.22 mg/l at age 4 weeks and 0.26 ± 0.11 mg/l at age 12 weeks) [19].

Aminoglycosides such as gentamicin are polycationic, highly polar and have poor lipid solubility, resulting in relatively low uptake by phagocytic cells [22]. Encapsulation in liposomes is one method by which the intracellular penetration of drugs might be enhanced. The *in vivo*

TABLE 1: Pharmacokinetic variables (mean ± s.d.) for gentamicin in plasma after i.v. administration of free gentamicin sulfate (FG) or liposomal gentamicin sulfate (LG) at a dosage of 6.6 mg/kg bwt to 8 foals (*Study 1*)

	Drug		
Variable	FG	LG	P
A (mg/l)	53.2 ± 25.8	28.8 ± 24.2	0.007
α (/h)	0.82 ± 0.28	0.76 ± 0.42	0.572
$t_{½α}$ (/h[1])	0.97 ± 0.45	1.10 ± 0.44	0.602
B (mg/l)	2.50 ± 1.50	1.49 ± 0.74	0.120
β (/h)	0.12 ± 0.04	0.04 ± 0.01	<0.001
$t_{½β}$ (h)	6.20 ± 1.77	16.3 ± 3.5	<0.001
Vd_{area} (l/kg bwt)	0.72 ± 0.32	2.00 ± 1.03	0.010
Vd_{SS} (l/kg bwt)	0.24 ± 0.11	1.09 ± 0.71	0.012
CL (ml/h/kg bwt)	85.2 ± 36.9	88.7 ± 45.5	0.822
AUC_{0-t} (mg•h/l)	96.7 ± 63.8	66.1 ± 18.1	0.362
$AUC_{0-\infty}$ (mg•h/l)	98.4 ± 64.3	71.9 ± 15.8	0.247
$AUMC_{0-\infty}$ (mg•h^2/l)	360 ± 364	968 ± 574	0.016
MRT (h)	3.18 ± 1.25	13.0 ± 4.4	<0.001
$C_{0.5h}$ (mg/l)	71.78 ± 92.14	19.14 ± 10.63	0.011
C_{1h} (mg/l)	32.60 ± 25.28	13.18 ± 4.40	0.040
C_{24h} (mg/l)	0.25 ± 0.19	0.50 ± 0.33	0.037
C_{48h} (mg/l)	0.09 ± 0.09	0.23 ± 0.16	0.012

A and α = intercept and rate constant, respectively of the distribution phase; $t_{½α}$ = distribution half-life; B and β = intercept and rate constant, respectively of the elimination phase; $t_{½β}$ = elimination half-life; Vd_{area} = apparent volume of distribution based on AUC; Vd_{SS}= apparent volume of distribution at steady state; CL = systemic clearance; $AUC_{0-\infty}$ = area under the plasma concentration vs. time curve extrapolated to infinity; $AUMC_{0-\infty}$ = area under the first moment of the concentration vs. time curve extrapolated to infinity; MRT = mean residence time; $C_{0.5h}$ = plasma concentrations of gentamicin 30 min after administration; C_{1h} = plasma concentrations of gentamicin 1 h after administration; C_{24h} = plasma concentrations of gentamicin 24 h after administration; C_{48h} = plasma concentrations of gentamicin 48 h after administration.

TABLE 2: Pharmacokinetic variables (mean ± s.d. unless otherwise specified*) for gentamicin concentration in bronchoalveolar (BAL) cells and pulmonary epithelial lining fluid (PELF) after i.v. or nebulised (Neb) administration of free gentamicin (FG) or liposomal gentamicin (LG) sulfate at a dosage of 6.6 mg/kg bwt to 8 foals (*Study 1*)

			Drug		P value		
Sample	Variable	Route	FG	LG	Drug	Route	Drug × route
BAL cells	C_{max} (mg/l)	i.v.	2.98 ± 1.67[a]	5.27 ± 2.67[b]	<0.001	0.076	0.472
		Neb	1.49 ± 0.57[a]	4.47 ± 2.66[b]			
	T_{max} (h)*	i.v.	2 (2–8)[a]	24 (2–48)[b]	0.028	0.809	0.433
		Neb	3 (2–48)[a]	4 (2–24)[b]			
	AUC_{0-t} (mg•h/l)	i.v.	58.9 ± 41.5[a]	145 ± 65[b]	<0.001	0.102	0.860
		Neb	37.2 ± 19.0[a]	114 ± 76[b]			
	C_{24h} (mg/l)	i.v.	1.50 ± 1.23	4.27 ± 3.30	0.087	0.118	0.307
		Neb	1.01 ± 0.57	2.52 ± 2.26			
	C_{48h} (mg/l)	i.v.	0.47 ± 0.62[a]	2.09 ± 1.47[a]	0.003	0.399	0.380
		Neb	0.32 ± 0.23[a]	1.26 ± 1.28[b]			
PELF	C_{max} (mg/l)	i.v.	4.64 ± 1.99[a]	1.21 ± 0.48[b]	<0.001	0.007	0.273
		Neb	13.0 ± 6.7[c]	2.05 ± 1.28[d]			
	T_{max} (h)*	i.v.	6 (4–8)	6 (2–24)	0.938	0.082	0.189
		Neb	2 (2-2)	4 (2–24)			
	AUC_{0-t} (mg•h/l)	i.v.	44.7 ± 21.0[a]	12.9 ± 6.4[b]	0.006	0.759	0.539
		Neb	41.0 ± 15.8[a]	17.1 ± 13.5[b]			
	C_{24h} (mg/l)	i.v.	0.84 ± 0.64	0.42 ± 0.60	0.371	0.119	0.457
		Neb	0.35 ± 0.34	0.74 ± 1.06			

*Median and range. C_{max} = maximum concentration; T_{max} = time to maximum concentration; AUC_{0-t} = area under the plasma concentration vs. time curve until the last measurable time point; C_{24h} = concentrations at 24 h; C_{48h} = concentrations at 48 h. [a,b,c,d]Different letters within a given variable indicate a statistically significant difference between drugs and/or administration route (P<0.05).

disposition of liposomes varies dramatically depending upon their specific lipid composition, particle size, and method of formulation, all of which affect the rate at which liposomes are taken up by mononuclear phagocytes and the extent to which they localise in affected tissues [9,16]. At the most basic level, liposomes can be divided into 2 main categories: conventional, short-circulating liposomes, which are composed of natural or synthetic phospholipids ± cholesterol, and long-circulating liposomes, sterically stabilised with high-phase transition lipids, cholesterol and polyethylene glycol (PEG) coating, which delay opsonisation and uptake by mononuclear phagocytes relative to conventional liposomes, thus resulting in prolonged systemic circulation time and higher tissue concentrations. A balance between uptake by phagocytic cells and stability in the circulation and at the site of infection must be achieved for therapeutic success. A sterically stabilised PEG-coated liposome formulation was developed for use in this study because of prior work showing significantly greater localisation of PEG-coated over conventional

TABLE 3: Mean (± s.d.) urinary gentamicin concentrations and selected plasma and urinary indices of renal injury on Days 0, 3 and 7 in foals receiving free gentamicin (FG; n = 6) or liposomal gentamicin (LG; n = 6) at a dose of 6.6 mg/kg bwt i.v. q. 24 h for 7 doses (*Study 2*)

		Time			P value		
Variable	Drug	Day 0	Day 3	Day 7	Drug	Time	Drug × time
Urine gentamicin (mg/l)	FG	–	94.8 ± 47.5	87.5 ± 5	0.130	0.376	0.746
	LG	–	66.2 ± 34.4	47.2 ± 53.0			
Plasma creatinine (μmol/l)	FG	116 ± 20	121 ± 21	118 ± 25	0.350	0.633	0.375
	LG	125 ± 7	122 ± 10	140 ± 36			
Urine protein/(creatinine x 0.001) ratio (g/mmol)	FG	23.9 ± 4.6	40.5 ± 48.5	34.7 ± 15.8	0.589	0.083	0.113
	LG	19.9 ± 9.5	20.5 ± 6.1	84.9 ± 91.3			
Urine GGT/(creatinine x 0.001) ratio (U/mmol)	FG	2.70 ± 1.62*	3.04 ± 1.26	8.75 ± 5.33	0.033	<0.001	0.376
	LG	1.38 ± 0.41*	2.26 ± 0.44	4.34 ± 1.86			
FE Na^+ (%)	FG	0.28 ± 0.4[a]	0.12 ± 0.05[a]	0.27 ± 0.12[b]	0.796	0.011	0.614
	LG	0.17 ± 0.17[a]	0.16 ± 0.07[a]	0.30 ± 0.18[b]			
FE K^+ (%)	FG	10.1 ± 4.9	18.4 ± 12.2	16.8 ± 10.6	0.721	0.067	0.632
	LG	13.0 ± 8.4	16.7 ± 8.9	20.6 ± 12.6			
FE Cl^- (%)	FG	0.60 ± 0.29	0.6 ± 0.2	0.78 ± 0.19	0.699	0.003	0.357
	LG	0.5 ± 0.2	0.53 ± 0.15	0.88 ± 0.3			
FE Mg^{2+} (%)	FG	13.9 ± 8.9	11.2 ± 6.5	9.8 ± 4.7	0.422	0.634	0.508
	LG	9.2 ± 4.4	9.5 ± 4.1	9.6 ± 6.4			
FE Ca^{2+} (%)	FG	3.5 ± 3.4	1.9 ± 1.1	1.6 ± 1.4	0.419	0.168	0.990
	LG	1.5 ± 1.0	1.3 ± 0.9	1.3 ± 1.5			
USG	FG	1.003 ± 0.002	1.008 ± 0.008	1.002 ± 0.001	0.906	0.291	0.107
	LG	1.008 ± 0.009	1.003 ± 0.002	1.003 ± 0.001			
Urine pH	FG	7.2 ± 0.7*	6.3 ± 0.8	6.3 ± 0.3	0.589	0.047	0.026
	LG	6.4 ± 0.4*	6.4 ± 0.6	6.5 ± 0.4			

GGT = γ-glutamyltransferase. FE = fractional excretion; USG = urine specific gravity. [a,b]Different letters within a given variable indicate a significant difference between days (P<0.05). *Indicate a significant difference between LG and FG on Day 0 (P<0.05).

TABLE 4: Mean ± s.d. difference from baseline (Day 0) in urine γ-glutamyltransferase (GGT):creatinine ratio and in urine pH in foals receiving free gentamicin (FG; n = 6) or liposomal gentamicin (LG; n = 6) at a dose of 6.6 mg/kg bwt i.v. q. 24 h for 7 doses (*Study 2*)

		Time		*P* value		
Variable	Drug	Day 3	Day 7	Drug	Time	Drug × time
Urine GGT/(creatinine × 0.001) ratio (U/mmol)	FG	0.34 ± 1.62[a]	6.05 ± 4.98[b]	0.556	<0.001	0.214
	LG	0.89 ± 0.38[a]	2.96 ± 1.66[b]			
Urine pH	FG	-0.83*	-1.67*	0.004	0.504	0.227
	LG	0.00*	0.25*			

[a,b]Different letters within a given variable indicate a significant difference between days (P<0.05). *Indicates a significant difference between LG and FG (P<0.05).

liposomes in the lungs of pneumonic rats and because of higher or similar efficacy of PEG-coated liposomal antimicrobials in animal models of bacterial infection [23,24].

The significantly longer plasma half-life exhibited by LG compared with FG after administration by the i.v. route in this study is consistent with the results of studies comparing liposomal vs. free aminoglycosides in laboratory animals [25]. The significantly longer plasma elimination half-life of LG in this study can be attributed to a significantly larger volume of distribution because systemic clearance was almost identical for both formulations. The significantly lower initial plasma concentrations and higher volume of distribution achieved after i.v. administration of LG are consistent with rapid uptake by phagocytes and distribution to tissues. The greater uptake of LG by phagocytes was confirmed by a significantly higher C_{max} and AUC in BAL cells after administration of LG compared with FG.

Aminoglycosides exert concentration-dependent bacterial killing characteristics. Their rate of killing increases as the drug concentration increases above the minimum inhibitory concentration (MIC) for a given pathogen with optimal maximum plasma concentration to MIC ratio of 8–10:1 [26,27]. The MIC that inhibits at least 90% (MIC_{90}) of *R. equi* isolates is 0.5 mg/l [28]. Although administration of both LG and FG resulted in peak concentrations of gentamicin in BAL cells above the MIC_{90} of *R. equi*, only i.v. or nebulised LG reached the optimal C_{max} to MIC ratio of 8–10:1. The advantage of liposomal formulations of gentamicin over FG in the intracellular environment may not be related solely to differences in intracellular concentration. Liposome formulations similar to the one used in the present study, have been shown to concentrate in phagosomes after engulfment by macrophages [11]. Thus, colocalisation of LG with bacteria in the phagosome could enhance intracellular killing of intracellular pathogens such as *R. equi*. Indeed, the LG formulation used in the present study was found to be superior to FG or to the combination of clarithromycin and rifampin in decreasing tissue colony forming units of *R. equi* in a mouse infection model [29]. Similarly, LG has been shown to be more effective than FG in animal models of infection with other facultative intracellular pathogens such as *L. monocytogenes, M. avium, Salmonella* spp. and *B. abortus* [10,12–16]. The advantage of LG over FG may not only apply to the treatment of intracellular pathogens. Infection models with extracellular pathogens such as *Klebsiella pneumoniae* have also shown an advantage of LG vs. FG [30].

Nebulised liposomal amikacin has been shown to be significantly more efficacious than nebulised free amikacin for the treatment of chronic *Pseudomonas aeruginosa* infection in rats and has been found to be safe and effective in human cystic fibrosis patients during Stage II trials [31,32]. In the present study, gentamicin concentrations in BAL cells were significantly higher after nebulisation of LG than after nebulisation of FG. Plasma concentrations of gentamicin were minimal after nebulisation with LG despite concentrations in BAL cells similar to those achieved after i.v. administration. Therefore, nebulisation of LG shows promise as an alternative to i.v. LG or concurrent administration by both routes could be used to further increase BAL cells and pulmonary concentrations of gentamicin with negligible contribution to systemic toxicity. Consistent with the greater cellular uptake of LG, concentrations of gentamicin in PELF were significantly higher after nebulisation with FG than after i.v. FG or after administration of LG regardless of route.

Liposomal encapsulation of drugs can minimise nontarget organ-specific drug toxicity but this is dependent upon interactions between liposome formulation, drug encapsulated, as well as rate and location of drug release [33]. The main adverse effect of gentamicin recognised in horses is nephrotoxicity resulting from tubular necrosis. No adverse effects were encountered with single dose i.v. or nebulised LG, and the incidence of adverse events (diarrhoea, thrombophlebitis) and indices of nephrotoxicity during repeated daily i.v. dosing were not significantly different between LG and FG. Urine GGT:creatinine ratio is a much more sensitive indicator of tubular damage than histopathology in mature horses with increases in urine GGT/creatinine ratio occurring after only 3–5 days of therapy with i.v. FG despite normal histopathology of the kidney [34,35]. Therefore, the increase in urine GGT/creatinine ratio observed after administration of LG or FG in the present study was not unexpected. Given that treatment of infections caused by *R. equi* typically requires a prolonged course of therapy [1], additional studies will be required to evaluate the safety of long-term administration of liposomal gentamicin.

In conclusion, administration of LG to foals by the i.v. or nebulised route is well tolerated and results in significantly higher intracellular concentration of the drug compared with what is achieved after administration of FG. Additional studies will be needed to evaluate the efficacy of this LG formulation for the treatment of susceptible bacterial pathogens of horses.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

The study was approved by the University's Institutional Animal Use and Care Committee.

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Authorship

A.J. Burton and S. Giguère were involved in all phases of the study. R.D. Arnold was involved in the study design, data analysis and interpretation, and manuscript preparation.

Manufacturers' addresses

[a]Avanti Polar Lipids Inc., Alabaster, Alabama, USA.
[b]Sigma-Aldrich, St-Louis, Missouri, USA.
[c]Buchi Corporation, New Castle, Delaware, USA.
[d]PCCA, Houston, Texas, USA.
[e]Avestin Inc., Ottawa, Ontario, Canada.

[f]Thermo Fisher Scientific Inc. Rockford, Illinois, USA.
[g]Brookhaven Instruments Corporation, Holtsville, New York, USA.
[h]Sparhawk Laboratories Inc., Lenexa, Kansas, USA.
[i]Nortev, Galway, Ireland.
[j]Malvern Instrument Limited, Malvern, Worcestershire, UK.
[k]Jorgensen Laboratories, Inc., Loveland, Colorado, USA.
[l]Nexcelom Bioscience, Lawrence, Massachusetts, USA.
[m]Biochain, Hayward, California, USA.
[n]Summit Research Services, Montrose, Colorado, USA.

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