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Intraosseous infusion of the distal phalanx compared to systemic intravenous infusion for marimastat delivery to equine lamellar tissue

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Highlights

- Ultrafiltration was used to collect lamellar interstitial fluid for pharmacokinetic analyses
- Intraosseous infusion of the distal phalanx (IOIDP) with marimastat results in similar lamellar marimaststst concentrations to systemic intravenous constant rate infusion.
- Further refinement of the IOIDP technique is necessary if it is to be useful for local lamellar drug delivery.

Abstract

No validated laminitis drug therapy exists, yet pharmaceutical agents with potential for laminitis prevention have been identified. Many of these are impractical for systemic administration but may be effective if administered locally. This study compared intraosseous infusion of the distal phalanx (IOIDP) with systemic intravenous constant rate infusion (CRI) to determine which was more effective for lamellar marimastat delivery. Ultrafiltration probes were placed in both forefeet of five horses to collect lamellar interstitial fluid as lamellar ultrafiltrate (LUF). Marimastat solution (3.5 mg/mL) containing lidocaine (20 mg/mL) was infused by IOIDP at 0.15 mL/min for 12 h. After a 12 h wash-out, marimastat (3.5 mg/mL) and lidocaine were infused by constant rate infusion (CRI) at 0.15 mL/min for 12 h. LUF, plasma and lamellar tissue marimastat concentrations were quantified using UPLC-MS. Zymography was used to establish the inhibitory concentrations of marimastat for equine lamellar matrix metalloproteinases (MMPs). Data were analysed non-parametrically.

There was no difference between the steady-state marimastat concentration in lamellar ultrafiltrate ($LUF_{[M]}$) during IOIDP (139[88-497] ng/mL) and CRI (136[93-157] ng/mL). During IOIDP, there was no difference between marimastat concentrations in the

treated foot (139[88-497] ng/mL), the untreated foot (91[63-154] ng/mL) and plasma (101[93-118] ng/mL). $LUF_{[M]}$ after IOIDP and CRI were $>IC_{50}$ of lamellar MMP-2 and 9, but below the concentration considered necessary for in vivo laminitis prevention. Lamellar drug delivery during IOIDP was inconsistent and did not achieve higher lamellar marimastat concentrations than CRI. Modification or refinement of the IOIDP technique is necessary if it is to be consistently effective.

Keywords: Horse; MMP-inhibitor; Pharmacokinetics; Laminitis; Ultrafiltration.

Introduction

Laminitis is a crippling disease of the equine foot. There are no experimentally validated pharmacological means of treating or preventing laminitis. Activation of matrix metalloproteinases (MMPs) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs)-4 has been implicated in inflammatory laminitis pathophysiology (Pollitt et al., 1998; Visser, 2009; Wang et al., 2012). The broad spectrum MMP inhibitors marimastat and batimastat prevent lamellar separation in vitro (Pollitt et al., 1998). They also inhibit ADAMTS-4 (Tortorella et al., 2009). Investigation of their in vivo efficacy is warranted, but has been prevented by their impracticality for systemic administration due to expense, systemic side effects (Levin et al., 2006), and rapid clearance after intravenous (IV) administration (M.A. Pass and C.C. Pollitt, unpublished data). A regional lamellar delivery technique is required for experimental evaluation of their anti-laminitis potential.

IOIDP has been investigated for short-term delivery of gentamicin to lamellar tissue (Nourian et al., 2010). Lamellar gentamicin concentrations were significantly higher than those in plasma, suggesting IOIDP had potential for regional lamellar delivery. However, administration of insulin by IOIDP over 48 h resulted in foot pain and no evidence of

effective local delivery (de Laat, 2011). Further investigation is warranted to determine if long-term IOIDP results in regional lamellar delivery and whether the technique is tolerated by the subject.

In this study, a modified intraosseous (IO) infusion of the distal phalanx (IOIDP) technique was compared with systemic constant rate infusion (CRI) to determine which delivered marimastat more effectively to the lamellar region of the horse's foot. Lamellar ultrafiltrate (LUF) obtained via lamellar ultrafiltration enabled sampling of lamellar interstitial fluid. The following hypotheses were tested: (1) IOIDP results in higher LUF marimastat concentrations ($LUF_{[M]}$) than CRI, and (2) $LUF_{[M]}$ in the foot receiving IOIDP are higher than those in the contra-lateral limb and in plasma ($P_{[M]}$). An additional objective was to further validate lamellar ultrafiltration for pharmacokinetic studies. Variations in $LUF_{[M]}$ and biochemical analyte (glucose, urea, sodium, potassium and chloride) concentrations over time, along with the difference between $LUF_{[M]}$ and lamellar tissue marimastat concentrations ($T_{[M]}$), were examined.

Materials and methods

The project was approved by the University of Queensland Animal Ethics Committee (AEC) (approval numbers SVS/337/11 and SVS/418/12) that monitors compliance with the Animal Welfare Act (2001) and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (current edition).

Animals and monitoring

Five Standardbred horses (four geldings, one mare; aged 6-11 years, 447-502 kg), with no lameness or gross foot abnormalities were enrolled in the study. During the

experiment the horses were housed in stalls with ad libitum access to hay and water, and monitored continuously. Heart rate, respiratory rate and pain behaviours were recorded at 0, 1, 2, 3, 4, 5, 6, 9 and 12 h during both IOIDP and CRI.

In vitro marimastat recovery

Stock solutions of marimastat (BB-2516, Vernalis) at 0.1, 0.5, 1, 10 and 100 µg/mL were prepared in pooled plasma and 0.9% saline; 200 µL of each stock solution were stored for analysis. Custom-made 3-8 ultrafiltration probes (BASi) were placed into 5 mL of each stock solution. Ultrafiltrate was collected for 2 h at 37 °C. Experiments were performed in triplicate and samples stored at -80 ^{0}C prior to analysis by ultra-performance liquid chromatography with tandem mass spectroscopy (UPLC-MS). Recovery was calculated according to the following equation:

$$\text{Recovery} = \frac{\text{concentration in ultrafiltrate}}{\text{concentration in initial solution}} \times 100$$

Zymography

To assess the potency and efficacy of marimastat on equine lamellar MMP-2 and 9, an in vitro gel zymography bioassay was used. Gelatin zymography was performed as previously described with minor variations (Pollitt et al., 1998; de Laat et al., 2011). Briefly, pooled lamellar tissue collected from horses 48 h after administration of 10 g/kg oligofructose (archived from a separate study) was homogenised (by pulverising the tissue to a powder in liquid nitrogen, and then re-suspending it in phosphate-buffered saline), centrifuged and the total protein concentration in the supernatant determined. Samples containing 10 µg protein were prepared and run on 7.5% acrylamide mini-gels containing 0.25% gelatine. Lanes were separately incubated with marimastat at different concentrations

(range 1 – 1000 ng/mL plus control) in gelatine refolding buffer for 24 h. Gels were stained then analysed by band densitometry (Unscan-it Software, Silk Scientific). Densitometry data were expressed as % of activity of control and experiments were repeated four times.

In vivo study design and sample collection

Twenty-four hours prior to IOIDP, ultrafiltration probes were placed in the lamellar region of each forelimb. IV catheters were placed in the left and right jugular veins (for blood collection and drug administration respectively). An IO cannula was placed in the distal phalanx of one forelimb on each horse (instrumented foot). Sterile marimastat solution (3.5 mg/mL marimastat, 20 mg/mL lidocaine [Ilium Lidocaine, Troy Laboratories] and 1.5 IU/mL heparin sodium [Pfizer]) were infused through the IO cannula with a target infusion rate of 0.25 mL/min.

Jugular blood and LUF from the instrumented foot were collected prior to marimastat administration, then at 1, 2, 3, 4, 5, 6, 9 and 12 h of infusion. LUF was collected from the contralateral, uninstrumented foot at 0, 6, 9 and 12 h. The volume of marimastat solution infused was recorded. After 12 h the IOIDP cannula was perfused with heparin and lidocaine solution for 1 h to remove residual marimastat then sealed. The horses underwent a 12 h wash-out period (12 × elimination half-life (59 ± 21 min) after systemic IV administration to horses (M.A. Pass and C.C. Pollitt, unpublished data) prior to CRI.

Baseline LUF samples were collected 1 h prior to CRI. An identical marimastat solution was administered by CRI through the right jugular catheter using microinfusion pumps at the same rate as the mean IOIDP infusion rate in that horse with sample collection time-points as for IOIDP. At the end of the CRI horses were euthanased with pentobarbital

sodium (Lethabarb, 20 mg/kg IV). Lamellar tissue encompassing the ultrafiltration probe was collected for histological and pharmacokinetic analyses.

Ultrafiltration probe placement

Sterile, custom-made ultrafiltration probes (3-8 UF) were placed in the lamellar region of both forelimbs under regional anaesthesia as previously described, with the minor variation that the probes were placed 1-2 cm lateral to dorsal midline (Underwood et al., 2014) The ultrafiltration tubing was cut to a length of 30 cm from the probe prior to sample collection. The location of the probe was marked on the surface of the hoof wall to ensure the probe was not damaged during IOIDP needle placement.

IOIDP needle placement

After sedation with 0.03 mg/kg acepromazine (A.C.P. 10, Delvet) and bilateral perineural anaesthesia of the palmar digital nerves at the fetlock and metacarpal nerves at the distal aspect of the 2nd and 4th metacarpal bones using mepivacaine (Ilium Vetacaine), the dorsal hoof was rasped and aseptically prepared. A midline site 25 mm below the hairline and 1-2 cm medial to the ultrafiltration probe was marked on the hoof wall. A 3.2 mm diameter hole was drilled perpendicular to the hoof wall. Drilling continued until the dorsal surface of the distal phalanx was contacted. The drill bit was adjusted so that further drilling extended 4 mm into the distal phalanx. The hoof wall was then over-drilled with a 4.8 mm drill bit.

A sterile 25 mm long nylon dummy needle with a bevelled tip was driven into the distal phalanx, flushed and withdrawn to remove any remaining debris. A sterile, custom made, IO needle (Fig. 1) formed from nylon tubing with an external diameter of 3.2 mm and a 1.6 mm hose barb elbow connector was primed with sterile heparinised saline, inserted into

the distal phalanx and tapped into place until the elbow was flush with the hoof surface. A 120 cm plastic extension tube (Pressure Tubing, Edwards) preloaded with sterile heparinised saline was connected to the needle and flushed. Hoof adhesive (Equilox) was applied to fix the tubing in place. Infusion with 3.5 mg/mL marimastat solution was initiated immediately using a Springfusor 30 pump and 30-2 Flow Control Tubing (Allied Medical).

Sample preparation and analysis

Marimastat concentrations were quantified in plasma, LUF and tissue homogenates using UPLC-MS on a Nexera UPLC coupled with a MS-8030 Triple quadruple mass spectrometer (Shimadzu) operating in positive electrospray ionization mode. All reagents were LC-MS grade. A reverse phase C18 column (Kinetex, 1.7 µm XB-C18, 100A, 50 × 2.1 mm, Phenomenex) was employed; the injection volume was 1 µL. The mobile phase consisted of water (Solvent A) and acetonitrile (Solvent B) in the following program: a gradient run of 5% B and 95% B for 3 min, held for 1 min and then run at 5% B in an isocratic mode for 2 min. The flow rate was maintained at 0.4 mL/min at a temperature of 50 °C. The drying gas was at 250 °C, the gas flow at 20 L/min, the nebulising gas flow at 3 L/min, and the heating block was at 400 °C. Argon was used as a collision gas, and the capillary voltage was 4.5 KV. A positive mode with MRM transmission of 332.20 → 86.15 was used. The dwell time was 100 ms, and the collision energy was set to -20 eV. The limit of quantification was 10 ng/mL.

Plasma and LUF samples were extracted using an acid extraction method. 5-sulfosalicyclic acid dihydrate (0.1 g/mL) was added in a 1:1 v/v ratio to the sample. The sample was vortexed for 30 s, centrifuged for 10 min at 15,625 *g* and the supernatant removed, added in a 1:1 v/v ratio to dimethylsulfoxide containing 500 ng/mL of Batimastat

(Vernalis) as an internal standard, vortexed, centrifuged (10 min, 15,625 *g*) and the supernatant removed for analysis. Tissue samples were homogenised in a 1:2 w/w ratio with water. 5-sulfosalicyclic acid dihydrate (0.1 g/mL) was added to the homogenate in a 1:1 w/w ratio. The samples were vortexed for 30 s and centrifuged for 10 min at 15,625 *g*. The supernatant was removed, added in a 1:1 v/v ratio to dimethylsulfoxide containing 500 ng/mL internal standard (Batimastat), vortexed, centrifuged (10 min, 15,625 *g*) and the supernatant removed for analysis. Calibration and quality control samples were obtained for each matrix (ultrafiltrate, plasma and tissue homogenate) by spiking the blank matrix with known amounts of marimastat. The calibration curves were analysed using linear regression with a minimum R^2 of 0.99. A measure of in vivo recovery of marimastat in LUF compared to tissue was calculated using the following equation:

$$\text{In vivo recovery} = \frac{\text{LUF}_{[\text{M}]}\ 9-12\ \text{h into CRI}}{\text{T}_{[\text{M}]}\ 12\ \text{h into CRI}} \times 100$$

Biochemical analyte concentrations were measured in LUF collected prior to IOIDP (0-12 h after probe implantation), between IOIDP and CRI (24-36 h post implantation) and during the last 6 h of the CRI (42-48 h post implantation), using a Beckman Coulter AU400 biochemistry analyser as previously described (Underwood et al., 2014).

Histological analysis

A lamellar tissue sample surrounding the ultrafiltration probe was fixed in 10% neutral buffered formalin, processed by routine paraffin embedding, sectioned at 4 µm and stained with H&E and Masson's Trichrome for light microscopy as previously described (Pollitt, 1996). The sections were interpreted by a blinded, specialist veterinary pathologist

(REA). The reaction around the ultrafiltration probe was scored using a previously described semi-quantitative method presented in Table 1 (Underwood et al., 2014).

Data analysis

The relationship between marimastat concentration and pharmacodynamic effect (MMP degradation of gelatin, expressed as % of control) was estimated using PKSolver, employing an inhibitory sigmoid E_{max} model in which no baseline (E_0) was applied as previously described (Shu et al., 2011). The maximum effect (E_{max}), the concentration required for 50% inhibition in vitro (IC_{50}) and the shaping factor (γ) were calculated for pro-MMP2, pro-MMP-9 and active MMP-2. IC_{80} and IC_{90} values were estimated for each protease. Three pharmacokinetic-pharmacodynamic indices were calculated, namely, steady state concentration (C_{ss}):$IC_{50,}$ C_{ss}:IC_{80} and C_{ss}:IC_{90}.

Data were analysed using GraphPad Prism 6.0. The data were tested for normality using D'Agostino-Pearson omnibus normality tests. Data distributions were either non-Gaussian, or data numbers were too small to presume a normal distribution, hence non-parametric tests were used. Paired data were compared by Wilcoxon signed-rank tests, and non-paired data by Mann-Whitney tests. Comparisons of repeated measures were analysed using Friedman analyses with Dunn's post-tests. Significance was set at $P \leq 0.05$. Unless otherwise stated, data are expressed as median (interquartile range).

Spearman's rank correlation coefficients (r_s) were calculated to examine the association between steady-state $LUF_{[M]}$ post IOIDP/CRI with LUF biochemical analyte concentrations and total histological scores. Data were also examined visually. A weak

correlation was defined as being significant (P<0.05) with an r_s <0.4, moderate 0.4–0.7, and strong >0.7 (Taylor, 1990).

Results

There was no difference between IOIDP and CRI infusion rates (0.15[0.14- 0.16] mL/min vs. 0.15[0.14-0.16] mL/min respectively). This equated to a marimastat infusion rate of 31.5 mg/h. Both infusions were well tolerated with no evidence of pain or lameness. There was no change in clinical parameters during either infusion.

The in vitro recovery of marimastat through the ultrafiltration probe was 98(95-100)% in 0.9% saline and 94(90-99)% in plasma. Ultrafiltration probes were placed in the lamellar tissue in both forefeet in all five horses. LUF was collected at 49(40-55) µL/h. There was no difference in LUF collection rates during IOIDP (52[39-65] µL/h) and CRI (47[40-54] µL/h), nor between the IOIDP instrumented foot (50[39-58] µL/h) and the uninstrumented foot (49[40-58] µL/h). The concentrations of biochemical analytes in LUF did not vary significantly during the study period (Table 2). Once steady state was reached, there was no significant difference in $LUF_{[M]}$ during IOIDP or CRI (Fig. 2A). $T_{[M]}$ 12 h into the CRI were significantly higher than $LUF_{[M]}$ obtained 9-12 h into the CRI (161[136-276] ng/g vs. 145[118-171] ng/g, respectively), resulting in an in vivo recovery of marimastat from tissue of 72(60-100)%. The within-limb coefficient of variation of marimastat concentrations was 5.9-32.9% in tissue samples and 1.5-6.7% in LUF.

$LUF_{[M]}$ and $P_{[M]}$ reached steady-state 2 h after initiation of IOIDP and CRI. There were no significant differences between $LUF_{[M]}$ or $P_{[M]}$ during IOIDP and CRI, nor between $LUF_{[M]}$ and $P_{[M]}$ after either IOIDP or CRI (Table 4, Fig. 2). There were no differences

between $LUF_{[M]}$ in the instrumented vs. the un-instrumented limbs during IOIDP or CRI. However, the $LUF_{[M]}$ in the instrumented limbs during IOIDP were highly variable and appeared to fall into two groups; two horses (horses 2 and 4, high LUF group) had higher steady-state $LUF_{[M]}$ (512[469-866] ng/mL) and the remainder (low LUF group) had lower steady-state $LUF_{[M]}$ (108[65-131] ng/mL, Fig. 3). Neither the $P_{[M]}$ after IOIDP nor the $LUF_{[M]}$ after CRI differed subjectively between these groups (but numbers were insufficient for statistical analysis).

Fig. 4 shows the in vitro inhibition of pro-MMP-2, pro-MMP-9 and active-MMP-2 by marimastat. PK-PD data are detailed in Tables 3 and 4. Histological examination of the tissue sections showed the probe situated between primary epidermal lamellae (PEL) in 7/10 limbs and within the sublamellar dermis in the remaining three horses. There was no significant difference in steady-state $LUF_{[M]}$ during CRI between probes located in the sub-lamellar dermis and those located between PEL. The histological changes around the probe were consistent with a mild foreign body response (Fig. 5). There was no significant correlation between total histological score and steady-state $LUF_{[M]}$ after IOIDP or CRI.

Discussion

IOIDP did not consistently result in higher lamellar marimastat concentrations than IV CRI, nor were the marimastat concentrations in the treated foot consistently higher than those in the untreated foot and plasma. Hence IOIDP was not considered a reliable method for local lamellar drug delivery and the hypotheses were rejected. These results are similar to those achieved when delivering insulin by IOIDP (de Laat, 2011), and with reports of IO infusion at sites other than the distal phalanx, where local delivery is generally not achieved unless a tourniquet is applied (Mattson et al., 2005; Errico et al., 2008) and IO infusion is

used as an alternative to systemic IV administration (Schalk et al., 2011). However, they differ from two previous investigations of IOIDP in horses where local lamellar delivery was successfully achieved (Nourian et al., 2010; de Laat et al., 2012).

The lack of local lamellar delivery in the present study may be attributable to variations in IO cannula placement technique (Nourian et al., 2010; de Laat et al., 2012) possibly causing the infusion to directly enter the systemic circulation. However, insulin delivery by IOIDP failed to achieve hyperinsulinaemia in veins of the treated foot (de Laat, 2011), despite using a previously successful placement method (Nourian et al., 2010; de Laat et al., 2012). Therefore further investigations are warranted to determine whether there is a specific placement site in the distal phalanx that results in local lamellar rather than systemic delivery. Biodistribution factors attributable to different physicochemical properties of the pharmaceuticals used may also be responsible for the variation in lamellar delivery during IOIDP (Bidgood and Papich, 2003).

Within this study the extent of lamellar delivery by IOIDP varied. In two horses local lamellar delivery with IOIDP appeared successful; in the remaining three horses there was no local delivery effect (Fig. 4). This may be attributable to several factors including variations in drug metabolism, IOIDP cannula placement, marimastat recovery and probe functionality. Probe factors were considered unlikely for the following reasons: (1) there was minimal difference between the in vivo recovery in the high LUF group (85[72-99]%) compared to the low LUF group (81[57-108]%); (2) the variations in in vivo marimastat recovery (56-108%) were insufficient to explain the inter-horse variability during IOIDP; (3) the $LUF_{[M]}$ during CRI and the LUF concentrations of biochemical analytes did not vary between groups, nor

315 did there appear to be any correlation between biochemical analyte and marimastat
316 concentrations.
317
318 Therefore, the inter-horse variations in $LUF_{[M]}$ during IOIDP probably reflect true
319 interstitial fluid marimastat concentration variations attributable either to inter-horse
320 variations in marimastat pharmacokinetics or variations in IOIDP cannula placement, which,
321 whilst undetectable to the authors, may have resulted in different degrees of lamellar
322 marimastat delivery. Further investigations, are required to determine whether this variation
323 occurs to the same extent in a larger population, and to establish its cause.
324
325 Activation of MMP-2, MMP-9, MMP-14, and ADAMTS-4 has been implicated in
326 inflammatory laminitis pathophysiology (Pollitt et al., 1998; Visser, 2009; Wang et al., 2012).
327 Zymography established basic pharmacodynamic data for marimastat against equine lamellar
328 MMP-2 and -9. Marimastat inhibits human MMP-14 and ADAMTS-4 with IC_{50} values of 0.5
329 ng/mL and 26.1 ng/mL, respectively (Peterson, 2006; Tortorella et al., 2009). Both CRI and
330 IOIDP yielded $LUF_{[M]}$ greater than the IC_{50} of MMP-2, MMP-9, MMP-14 and ADAMTS-4.
331 Direct extrapolations from IC_{50} data should, however, be interpreted with caution: frequently
332 concentrations much higher than the IC_{50} are required for adequate inhibition in vivo (Lees et
333 al., 2004; Peterson, 2006).
334
335 Marimastat concentrations required to inhibit lamellar MMPs in vivo are unknown. A
336 high level of enzymatic inhibition is generally required to produce clinical responses by
337 MMP-inhibitors, therefore, when available, IC_{90} concentrations were selected to represent a
338 therapeutic level of MMP inhibition (Lees et al., 2004; Shu et al., 2011). There are no
339 published IC_{90} values for MMP-14 and ADAMTS-4. Target trough plasma concentrations six

times the IC_{50} were employed when the anti-neoplastic activity of marimastat was evaluated (Millar et al., 1998). Based on this, target concentrations of at least 3 ng/mL and 156 ng/mL marimastat were set for inhibition of MMP-14 and ADAMTS-4, respectively (Peterson, 2006; Tortorella et al., 2009). IOIDP and CRI yielded steady-state $LUF_{[M]}$ greater than the target concentrations for MMP-2 and MMP-14 in all horses. However, neither technique consistently achieved target $LUF_{[M]}$ for MMP-9 and ADAMTS-4. Therefore it is questionable whether, at the rates used in this study, either technique would sufficiently inhibit lamellar MMPs in vivo.

IOIDP was performed without complication in all five horses. IOIDP in horses has previously been associated with signs of pain (pawing and weight shifting) (de Laat, 2011). Pain associated with IO infusion is also reported in people and lidocaine administration is recommended to counteract this (Schalk et al., 2011). The horses in our study received lidocaine to block pain associated with IOIDP. As co-administration with lidocaine may have altered the pharmacokinetics of marimastat, lidocaine was also infused during CRI. The results of this study indicate IOIDP with lidocaine did not result in noticeable foot pain when the horses were free to move in the stall; further lameness evaluation would be necessary to declare the technique 'pain free'.

This is the first study to describe the use of ultrafiltration to collect LUF for pharmacokinetic analyses and compare simultaneous tissue and ultrafiltrate concentrations. An additional objective was to validate lamellar ultrafiltration for pharmacokinetic studies. LUF was successfully collected from every probe placed. Probes were well tolerated and functioned for the duration of the study. LUF collection rate did not vary significantly with time, and was sufficient for analysis. Based on comparison of $T_{[M]}$ and $LUF_{[M]}$ at the end of

the CRI, in vivo marimastat recovery was 72%. A <100% in vivo recovery rate is expected as 'in vivo recovery' is a comparison between unbound drug concentrations in LUF and total drug concentrations in tissue, not a direct measure of probe function (Bidgood and Papich, 2003). The in vitro variability in marimastat recovery was minimal (range 90-102%); whereas inter-horse variability in 'in vivo recovery' (LUF vs. tissue marimastat concentration) was high (range 56-108 %). This was attributed to the high within-limb variation in $T_{[M]}$, ascribed to inaccuracies during the complicated homogenisation and extraction process, and reflects a limitation of using tissue homogenates in pharmacokinetic studies.

Key concerns with ultrafiltration include whether drug recovery remains constant over time or is affected by tissue response to the ultrafiltration probes. The histological findings in this study support those in a previous report, with mild inflammation and fibrous tissue formation around the probes (Underwood et al., 2014). These changes may alter drug recovery (Wisniewski et al., 2001). In the present study, there were no significant differences in $LUF_{[M]}$ during steady state, suggesting marimastat recovery was consistent. The lack of significant temporal variations in LUF biochemical analyte concentrations and any correlation between biochemical analyte concentrations or steady-state $LUF_{[M]}$ and total histological score suggests host tissue response had minimal effect on recovery.

A major limitation of this study was the small number of horses used. Initially, power calculations were performed to ensure sample size was sufficient whilst minimising the number of horses used in accordance with the welfare principles governing animal research (NHMRC, 2013). However, the high variability in $LUF_{[M]}$ after IOIDP was not anticipated. During IOIDP, the infusion rate varied very slightly in each horse (range 0.13- 0.17 mL/min),

presumably due to inter-horse variations in resistance to infusion. However, CRI was performed after IOIDP to ensure CRI infusion rates were matched to the IOIDP rate in each horse.

Conclusions

Both IOIDP and CRI of marimastat at a rate of 31.5 mg/h yielded sufficient $LUF_{[M]}$ to inhibit 50% of MMP-2, MMP-9, MMP-14 and ADAMTS-4 in vitro, and hence may have potential for laminitis prophylaxis. However, neither technique resulted in consistent local lamellar delivery at concentrations considered sufficient for effective in vivo inhibition of MMP-9 and ADAMTS-4. Based on the results of this study, CRI is more suitable for lamellar marimastat delivery as it is simpler, yields similar median lamellar marimastat concentrations and has potential to deliver high volumes of pharmaceutical. The CRI dose requires optimisation to ensure target lamellar marimastat concentrations are achieved prior to application in experimental studies evaluating the efficacy of marimastat as a laminitis prophylactic. However, CRI is a systemic delivery technique and even its experimental use may be precluded by cost so further investigation of alternative local delivery mechanisms, such as retrograde IV infusion of the distal limb under tourniquet, is warranted. If IOIDP could be optimised to consistently yield the higher rate of lamellar delivery achieved in 2/5 horses in this study it may be worthy of further development. Lamellar ultrafiltration played a key role in supplying samples of LUF for pharmacokinetic analysis. The successful use of lamellar ultrafiltration in this study demonstrated its potential for use in further pharmacokinetic studies investigating lamellar drug delivery.

Conflict of Interest Statement

None of the authors of this paper have a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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506 **Figure Legends**

507 Fig. 1: The custom made intraosseous infusion needle and 25 mm long flushing needle used
508 to remove debris from the holes drilled in hoof and bone.

509

510 Fig. 2: Median (±IQR) marimastat concentrations during intraosseous infusion of the distal
511 phalanx (IOIDP) and systemic continuous rate infusion (CRI) with marimastat. (A) Lamellar
512 ultrafiltrate marimastat concentrations ($LUF_{[M]}$) during IOIDP (black circles) and CRI (grey
513 squares). (B) Plasma marimastat concentrations ($P_{[M]}$) during IOIDP (black circles) and CRI
514 (grey squares). (C) $P_{[M]}$ (grey triangles) and $LUF_{[M]}$ (black circles) during IOIDP. (D) $P_{[M]}$
515 (black triangles) and $LUF_{[M]}$ (grey squares) during CRI. There were no significant differences
516 between any of the concentrations at any time-points.

517

518 Fig. 3: Box and whisker plot showing steady-state ultrafiltrate marimastat concentrations in
519 each horse during IOIDP.

520 Fig. 4: (A) Zymography of a lamellar homogenate pooled from horses with oligofructose-
521 induced laminitis. MMP inhibition was achieved by incubating individual gel lanes with
522 increasing concentrations of marimastat. (B) % inhibition of lamellar MMP gelatin
523 degradation by increasing concentrations of marimastat.

524 Fig. 5: Median (±IQR) histological scores of lamellar sections surrounding the ultrafiltration
525 probe.

526

527 Table 1: The semi-quantitative histological scoring system applied to the lamellar slides.

Score	**0**	**1**	**2**	**3**
Epidermal basal cell and parabasal cell hyperplasia: expressed as fold increase in	Normal	2 fold	3 fold	≥ 3 fold

thickness of epidermal cell layer				
Flattening of secondary epidermal lamellae (SEL): expressed as % of length of unaffected SEL remaining	100%	66-99%	50-66%	<50%
Mitotic figures: per high power field at 200x magnification in the most affected area	0	1	2	≥3
Inflammatory cell count surrounding the probe	0	1-50	50-100	>100
Fibroplasia: thickness of the fibrous tissue around the probe compared to the width of unaffected primary epidermal lamellae (PEL)	0	<1 PEL width	1 PEL width	>1 PEL width
Collagen bundle formation	Absent	Mild	Moderate	Marked
Cellular debris	Absent	Mild	Moderate	Marked
Endothelial reactivity: the number of vessels with reactive endothelium around the probe	0	1-20	21-50	>50
Lamellar necrosis: the number of necrotic PEL	0	1	2	≥3

528
529

530 Table 2: The results of the biochemical analyses of ultrafiltrate through the study, data are
531 reported as median (IQR). There were no significant differences in analyte concentrations at
532 any of the time points.

Analyte	0-12 h post probe placement	24-36 h post probe placement	42-48 h post probe placement
Urea (mmol/L)	7.0 (5.7-8.6)	6.1 (5.0-7.6)	5.6 (5.4-6.4)
Glucose (mmol/L)	2.9 (0.7-4.2)	3.2 (0.7-3.9)	2.7 (0.0-3.2)
Sodium (mmol/L)	137 (134-140)	131(129-134)	136 (133-138)
Potassium (mmol/L)	4.2 (4.0-4.4)	3.9 (3.9-4.3)	4.2 (4.1-4.4)
Chloride (mmol/L)	106 (104-110)	103 (102-104)	107 (104-108)

533
534

535 Table 3: Estimates of pharmacodynamic parameters for marimastat inhibition of equine
536 lamellar matrix metalloproteinases (MMPs) MMP-2 and MMP-9 in vitro.

	Pro-MMP-9	Pro-MMP-2	Active-MMP-2
E_{max} (%)	102.8 (97.2-106.6)	114(105-119)	105(103-109)
γ	0.75(0.65-1.13)	0.64(0.62-0.92)	0.9(0.7-1.3)
IC_{50} (ng/mL)	9.2(7.4-18.8)	2.8(1.6-3.5)	2.5(2.0-3.6)
IC_{80} (ng/mL)	66(54-79)	25(16-38)	20(20-20)
IC_{90} (ng/mL)	177(131-260)	81(38-116)	40(18-56)

537

538 E_{max}, maximum effect; IC_{50}, concentration required for 50% inhibition in vitro; γ, shaping
539 factor.

Table 4: Estimates of pharmacokinetic-pharmacodynamic parameters for MMP-2 and MMP-9 after marimastat administration by intraosseous infusion of the distal phalanx (IOIDP) and systemic constant rate infusion (CRI). Data expressed as median (IQR)

	IOIDP LUF	IOIDP plasma	CRI LUF	CRI plasma
C_{ss} (ng/mL)	139(88-497)	103(69-114)	136(93-157)	105(85-156)
C_{ss}:$IC_{50\text{-}MMP9}$	15(9.0-55)	14(9.0-14)	11(9.1-18)	11(2.1-7.7)
C_{ss}:$IC_{90\text{-}MMP9}$	0.8(0.5-2.9)	0.7(0.5-1.0)	0.6(0.5-0.7)	0.6(0.5-0.9)
C_{ss}:$IC_{50\text{-}MMP2}$	50(29-181)	47(30-63)	37(30-45)	37(30-59)
C_{ss}:$IC_{90\text{-}MMP2}$	1.7(1.0-6.3)	1.6(1.0-2.2)	1.3(1.0-1.6)	1.3(1.0-2.0)
C_{ss}:$IC_{50\text{-}MMP2\_active}$	56(33-203)	53(33-71)	41(33-51)	41(34-66)
C_{ss}:$IC_{90\text{-}MMP2\_active}$	3.5(2.1-12.7)	3.3(2.1-4.4)	2.6(2.1-3.2)	2.5(2.1-4.1)

LUF, lamellar ultrafiltrate, C_{ss}, steady-state concentration (ng/mL).

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