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Correspondence: Dr Zimei Wu,
School of Pharmacy,
The University of Auckland,
Auckland, New Zealand.
E-mail: z.wu@auckland.ac.nz

Research Paper

Tissue compatibility and pharmacokinetics of three potential subcutaneous injectables for low-pH drug solutions

Zimei Wu[a], Ian G Tucker[b], Majid Razzak[c], Keith McSporran[d] and Natalie J Medlicott[b]

[a]School of Pharmacy, University of Auckland, New Zealand; [b]School of Pharmacy, University of Otago, New Zealand; [c]PharmTech Service Ltd, Auckland, New Zealand and [d]Gribbles Veterinary, Auckland, New Zealand

Abstract

Objectives The aim of the study was to investigate the tissue tolerance and bioavailability of four formulations containing 5% ricobendazole solubilised at low pH, following subcutaneous injection in sheep. Formulations were: a water-in-oil emulsion, a microemulsion, a hydroxypropyl-β-cyclodextrin (HP-β-CD, 20%) drug solution, and a low-pH drug solution (reference).
Methods In-vitro cytotoxicity of the formulations was investigated in L929 fibroblasts using MTS viability and lactate dehydrogenase leakage assays. Each formulation and respective vehicle was injected into either side of the back of a sheep to investigate the tissue tolerance and pharmacokinetics.
Key findings In-vitro studies suggested that both the emulsion and the microemulsion are unlikely to give a burst release of the low-pH drug solution in aqueous media. The microemulsion showed the greatest in-vitro cytotoxic effect but no significant difference was observed between the other formulations. In sheep, the three new formulations and vehicles caused little or no injection-site reactions compared with a marked response to the reference formulation. Bioavailabilities of HP-β-CD formulation, emulsion and microemulsion formulations, relative to the reference formulation, were 194, 155 and 115%, respectively.
Conclusions The three new subcutaneous injectables showed promise for reducing irritation of low-pH solubilised ricobendazole. HP-β-CD significantly enhanced the drug absorption. Controlling the burst release of the low-pH drug solution may improve tissue tolerance and minimise post-injection precipitation, and hence increase drug bioavailability. The in-vitro cytotoxicity studies did not predict the in-vivo irritation effects.
Keywords bioavailability; controlled release; cytotoxicity; poorly soluble drug; post-injection precipitation; tissue tolerance

Introduction

Effective and safe delivery of poorly water soluble drugs has always been a challenge in parenteral formulation development because these formulations must usually be optimised not only for solubility but also stability, injectability and tissue tolerability.[1] It has been estimated that approximately 10% of drugs currently on the market have a solubility problem,[2] and 40% of drug substances in current development are poorly water soluble.[3] Co-solvency, pH alteration or a combination of these methods are often used to increase solubility. However, there is a concern associated with these solubilisation approaches that these formulations often lead to products that cause irritation and injection site reactions.[4–8] Pain and tissue damage may occur as a result of irritation or as a delayed immune or inflammation response. Furthermore, for some poorly soluble drugs, even if sufficient solubility is achieved in a non-toxic injectable vehicle, post-injection precipitation of drug may occur at the injection site.[9–13] Precipitation at the injection site is likely to cause problems in several ways:[11] (i) mechanical irritation or blockage of the local blood vessels caused by the particles of the precipitated drug, (ii) local cytotoxicity at the injection site due to the prolonged drug–tissue contact time and (iii) poor and erratic systemic absorption due to redissolution of the precipitated drug.[13]

A number of formulation approaches have been investigated to reduce irritancy of injectables. Emulsions have been considered as a drug-delivery carrier, when the irritant

drug can be incorporated into the inner phase.[14] Another advantage of emulsion systems over co-solvent-based formulations is that following injection of an emulsion, drug precipitation is less likely.[15] Microemulsions have also received increasing interest as parenteral delivery systems for poorly water-soluble drugs, particularly those prepared using non-ionic surfactants, because of their high solubilising capacity, thermodynamic stability and ease of manufacture.[16–21] Reduced pain on injection in a rat paw-lick test was reported for clonixic acid when it was incorporated into the inner-phase of an oil-in-water microemulsion.[21] Apart from their effect on solubility enhancement, cyclodextrins (CDs), particularly hydroxypropyl-β-cyclodextrin (HP-β-CD) and sulfobutylether-β-cyclodextrin (SBE-β-CD), have also been used to mask irritation of parenteral formulations through formation of inclusion complexes.[22–26] This approach has an advantage over co-solvency or pH adjustment approaches, as drug precipitation at the injection site is often reduced or absent.[10,25–29]

Ricobendazole (RBZ) is the most active metabolite of albendazole, a benzimidazole anthelmintic used in veterinary practice for the control of gastrointestinal (GI) worms. It is currently available as a 10 or 15% w/v solution for injection and the pharmacokinetics of these subcutaneous (s.c.) injections have been reported.[30–33] The injection is formulated as a highly acidic solution with additional solubilisation by organic co-solvents and is used at a dosage of 3.75–7.50 mg/kg. Following s.c. administration, RBZ is rapidly absorbed and extensively distributed to the GI tract, which is favourable for clinical efficacy. However, pain on injection and low bioavailability of around 40% are reported.[30,33] This poor bioavailability may be due to drug precipitation at the injection site followed by slow and incomplete redissolution. One of the studies did report the appearance of drug precipitation at the injection site.[33] Hence, better tolerated formulations with improved bioavailability are required.

Our preformulation work[34] showed that RBZ is practically insoluble in water (62 μg/ml) or pharmaceutical oils (log P = 1.2). Sufficient solubility (>50 mg/ml) could not be achieved by co-solvency or complexation with HP-β-CD unless the pH was lower than 1.5 ($pK_a = 3.5$), resulting in likely tissue irritation on injection. However, further in-vitro studies suggested that, while the low-pH RBZ aqueous solutions readily precipitated on dilution with buffer pH 7.4, addition of HP-β-CD produced a concentration-dependent inhibition of the drug precipitation.[35]

In this study, a water-in-oil (w/o) emulsion, a microemulsion and a cyclodextrin formulation were developed for delivering RBZ solubilised at low pH. It was envisioned that these formulations may create favourable microenvironments at the injection site to minimise injection site reactions. The emulsion and microemulsions would provide an oily non-irritating barrier between the tissue and low-pH drug solution and reduce drug precipitation. The HP-β-CD formulation would result in decreased drug precipitation at the injection site. This paper reports on the cellular compatibility of these three formulations in an L929 fibroblast cell model, and the tissue tolerance and relative bioavailability following s.c. administration in sheep. A 5% w/v RBZ acidic solution (pH ~ 1.5) served as a reference formulation.

Materials and Methods

Reagents

Ricobendazole (99.3%) was a gift from Transchem Limited, Ambernath, India. Ethyl oleate was purchased from Inoue perfumery Mfg Co Ltd, Japan. Palsgaard 4125 (polyglycerol polyricinoleate, PGPR) was a gift from Palsgaard A/S, Denmark. Span 80, also known as sorbitan monooleate, was kindly donated by Croda (Croda Singapore Ltd). Both Labrafac CC, a commercial medium chain triglyceride (MCT), and Labrasol (caprylocaproyl macrogol-8-glycerides) were kindly donated by Gattefossé (France). Solutol HS 15 (macrogol-15-hydroxystearate), used in microemulsion development, was a gift from BASF Ltd. HP-β-CD was kindly provided by Roquette (France). The other reagents, all of AR grade, were from BDH Ltd (Poole, UK).

Formulation preparation

Formulations containing 5% w/v (0.18 M) RBZ were prepared by solubilising RBZ in HCl solutions at a molar ratio of 3 : 4 before incorporating into the vehicles. The emulsion was prepared by hand-shaking 55 : 42 : 3 (v/v/v) RBZ acidic solution : ethyl oleate : non-ionic emulsifiers (a 1 : 1 blend of PGPR and sorbitan monooleate). The microemulsion comprised 20% w/v of a concentrated RBZ solution (25% w/v), 20% w/v Labrafac CC and 60% w/v surfactants (sorbitan monooleate, Solutol HS 15 and Labrasol at 2 : 3 : 4 (w/w/w)). It was prepared by shaking the drug solution and the oil/surfactant mixture until a clear microemulsion was formed. The cyclodextrin formulation containing 20% w/v HP-β-CD was prepared by dissolving RBZ in the acid solution (pH 1.5) and adding HP-β-CD before making up to volume with water for injection. The respective vehicles were prepared using the same methods, with the drug solutions replaced by water for injection. A 5% w/v (0.18 M) RBZ acidic solution (pH ~ 1.5) in 0.2 M HCl served as a reference formulation.

For the sheep study, formulations and vehicles were prepared using an aseptic technique in a laminar-flow cabinet the day before injection. Sodium chloride injection 0.9% was used as negative control for in-vitro and in-vivo irritation assessments.

In-vitro characterisation of the formulations

The viscosity of the w/o emulsion and the microemulsion were measured using a Brookfield DV-III viscometer. The droplet size of the w/o emulsion was determined by laser diffraction using a Mastersizer-X (Malven, UK).

Entrapment of the acidic solution by the formulations

To assess entrapment of the hydrogen ions (H^+) by the vehicles, formulations were titrated into 100 ml Sörenson's phosphate buffer (SPB, 80 mM, pH 7.4) with constant stirring by a magnetic bar. The changes in pH of the buffer over the titration were monitored.

Microscopic examination

To visualise the emulsion/buffer and microemulsion/buffer interfaces, samples were examined using a light microscope (Nikon, Watson Victor Ltd, Japan) coupled with a digital camera. Slides were prepared by placing a small drop of the

formulation next to a small drop of buffer and the interface was observed when the cover slip was mounted and two drops began to merge.

In-vitro cytotoxicity assays

The cytotoxicity of the formulations and vehicles was estimated in cell culture. L929 fibroblasts cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Auckland), 3 mM glutamine and 100 U/ml penicillin G, and 100 μg/ml streptomycin (Gibco, Auckland) at 37°C in 5% CO_2, 95% air. The medium was changed every 2 days. The cells were harvested with 0.25% trypsin-EDTA (Gibco, Auckland) when confluent and passaged with a split of 1 : 6. Cells, between passages 5 and 13, were seeded into a 96-well plate at a density of 6400 cells/100 μl per well and incubated for 24 h before the cytotoxicity assays.

Formulations and the respective vehicles were diluted with normal saline (pre-warmed at 37°C) 1 in 200 for short-term (2 h) cytotoxicity testing and 1 in 1000 for long-term (20 h) cytotoxicity, and then filtered through a sterile filter (0.45 μm) before adding into cells at 100 μl/well. After incubation, cells were washed with sterilised saline before cytotoxicity assays were conducted. Cell viability was assessed by MTS assay using a commercial assay kit, CellTiter 96 AQ_{ueous} One Solution Reagent (Promega, Madison, USA) with the manufacturer's method. For the lactate dehydrogenase (LDH) assay, cells were lysed with 0.8% Triton X-100 before measuring the LDH activity using a CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Madison, USA). Spectrophotometric readings were performed using a SpectraMax Plus microtitre plate reader with SoftMax Pro software (Version P1.12, Molecular Devices) for all the assays. Cytotoxicity was expressed as the percentage of control (normal saline). In both assays, the background absorbance of the medium controls was subtracted from the sample reading.

Sheep study

Animal studies were approved by the Animal Ethics Committee, University of Otago. Five female sheep weighing 69.1 ± 3.4 kg in good health were used. They received no injections during the preceding 6 weeks and had no preexisting visible lesions at the injection sites.

Drug administration and collection of blood samples in sheep

On the day before injection of the test formulations and vehicles, a 14-gauge 5.1-cm intravenous catheter was implanted in the jugular vein under anaesthesia (Acezine 0.2 mg/kg and Ketalar 2 mg/kg, i.v.). Injection sites, 10 × 20 cm, on each side of the dorsal midline were shaved. After recovery for 24 h, each animal received s.c. administration of one of the test formulations (HP-β-CD formulation, emulsion, microemulsion, reference formulation or normal saline) on one side of the back and the respective vehicle on the contralateral side (the side was randomly assigned). Formulations and vehicles were administered to the sheep by a veterinarian using 19 gauge needles at a dose of 0.1 ml/kg body weight (equivalent to RBZ 5 mg/kg). Blood samples (5 ml) were withdrawn via jugular catheter 5 min pre-injection and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 9, 12, 18, 24, 30, 36, 42, 48 and 72 h post-injection for assay of RBZ and creatine kinase (CK). Samples were stored in heparinised tubes before the plasma was separated by centrifugation at 3000 rev/min for 10 min and stored in Eppendorf tubes at –20°C until analysed.

Visual observation of injection sites

Visual observations were made to assess tissue reactions by noting: (i) signs of pain on injection (backward movements and back stiffening), (ii) swelling and redness at the injection site and (iii) animal response to palpation of the injection sites. Responses were graded as (–) no obvious reaction, (±) mild or transient reaction and (+) significant reaction.

Plasma creatine kinase

To evaluate the tissue damage to the underlying muscles, plasma CK concentrations were measured before and at 1, 3, 6, 9, 12, 18 and 24 h and again on days 3, 4, 5, 6 and 7 after injection. The analysis was performed by Gribbles Veterinary Pathology Laboratories (Dunedin, NZ) using a commercial ELISA assay kit (Roche Diagnostics, Auckland, NZ).[36]

Skin temperature at the injection site

Skin temperatures at the injection sites were measured before and at 1, 2, 4, 6, 9, 12, 18, 24, 30, 36, 48, 60 and 72 h post-injection using an infrared non-contact thermometer (model 42530, Extech instruments Corp, Waltham, USA). The accuracy and resolution of the thermometer were ±2% of reading and 0.1°C, respectively. Maximum skin temperature, Tem_{max}, and the time it occurred, t_{max}, were used as descriptors of the tissue inflammatory reaction.

Tissue histology at the injection site

At 14 days post-injection, sheep were anaesthetised by a slow intravenous injection of Acezine (0.2 mg/kg) and a local injection of Nopaine (2% lignocaine). Then tissue samples were biopsied using a 12 mm punch to the depth of the hypodermis. Specimens were placed in 10% formalin and embedded in paraffin. Micrometre thick sections were stained with haematoxylin and eosin (H&E). Histological examination was performed by Gribbles Veterinary Pathology (Auckland, NZ), who were blinded to the treatments.

Pharmacokinetics of ricobendazole following subcutaneous injection

A validated reversed-phase HPLC method was used for quantification of RBZ in the plasma samples after solid-phase extraction as described previously.[37] The limit of quantification by this HPLC assay was 7.1 ng/ml for RBZ. Pharmacokinetic parameters were estimated using non-compartmental analysis based on statistical moment theory.[38] The maximum concentration C_{max} and the time when it occurred, T_{max}, were observed directly. The linear trapezoidal rule was used to calculate the area under the curve (AUC). AUC extrapolated beyond the last data point C_n to infinite time was obtained from C_n/λ using equation 1:

$$\begin{aligned} AUC_{0\sim\infty} &= AUC_{0\sim t} + C_n/\lambda \\ &= \sum_{i=1}^{n} \frac{C_i + C_{i+1}}{2} \cdot (t_{i+1} - t_i) + C_n/\lambda \end{aligned} \quad (1)$$

where λ is the slope obtained from the regression of natural log concentration versus time in the terminal phase, over the last three data points. The elimination rate constant k was estimated based on λ, and the terminal half-life ($t_{1/2}$) was calculated as $t_{1/2} = 0.693/k$. Relative bioavailability (F) of different formulations compared to the reference formulation was estimated by comparison of $AUC_{0-\infty}$ values. Data analysis was performed using PRISM software (GraphPad version 4.01).

Statistical analysis

Analysis of variance (ANOVA) or *t*-tests were performed using Minitab for Windows, version 12.1 (Minitab, Inc. PA, USA), with $P < 0.05$ considered statistically significant.

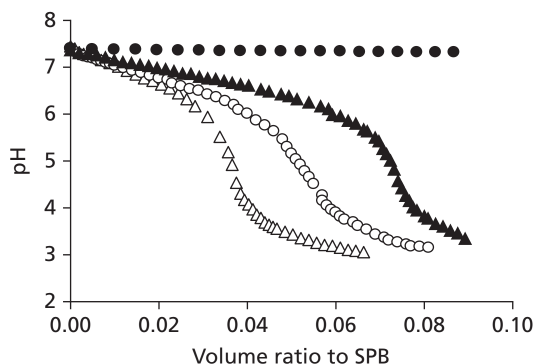

Figure 1 Comparison of the pH changes of Sörenson's phosphate buffer on titration with different formulations containing 5% w/v ricobedazole (solubilised in low pH). △, reference formulation (aqueous low pH solution); ▲, HP-β-CD formulation; ○, microemulsion (ME); ●, w/o emulsion. SPB, Sörenson's phosphate buffer.

Results

In-vitro characterisation of the formulations

The microemulsion showed Newtonian flow and the viscosities of the microemulsion at 25 and 37°C were 182 and 106 mPa.s, respectively. The emulsion could be reproducibly formed by hand-shaking the components for 2 min and had a mean droplet size of 2.5 μm with D (v, 0.9) <5 μm. The formulation appeared physically stable for at least 7 days at 37°C and 6 months at 5°C, with no sign of phase separation. Microscopy observation showed that the emulsion droplet size slightly increased in 3 days at 37°C and 7 days at 5°C. The emulsion showed a slight plastic character with low yield values (~1 mPa) and plastic viscosities 45–50 mPa.s under a shear rate range 10–40 s^{-1} at 30°C, which translated into good syringability.

Entrapment of the acidic solution by the formulations

Figure 1 shows the alteration in pH of the buffer on titration by the formulations. The results demonstrate a slow release of H^+ from all three formulations compared with the reference formulation.

Microscopic examination

Light microscopy observation of the emulsion/buffer interface showed emulsion droplets moving around the interface, the movement being restricted to the emulsion oil phase with no escape into the buffer, probably due to the interfacial tension (Figure 2). There was evidence of formation of w/o/w droplets at the interface but no evidence of droplet rupture. Droplet rupture could, however, be induced by addition of 10% v/v ethanol to the buffer.

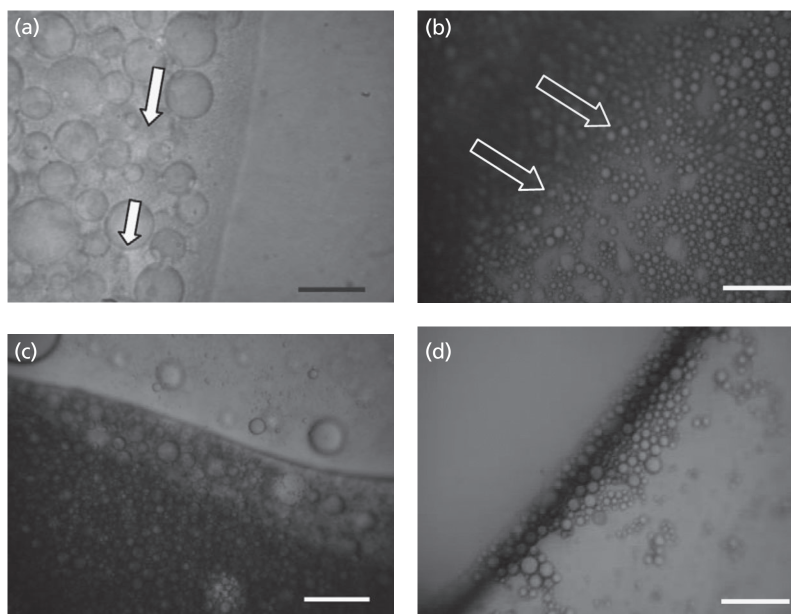

Figure 2 Interface between Sörenson's phosphate buffer and water/oil emulsion or microemulsion. Interface between SPB (containing Indigo carmine, left) and w/o emulsion or microemulsion (containing oil soluble dye, Sudan III, right). (a), emulsion/SPB interface at 0 h (bar = 10 μm) and (b), approximately 2 h. Droplet rupture of the emulsion was observed when 10% ethanol was present in the buffer (c) and microemulsion/buffer interface (d). Bar = 40 μm. SPB, Sörenson's phosphate buffer.

For the microemulsion, a coarse emulsion layer at the interface between the buffer and formulation was observed, consistent with a self-emulsifying action of the microemulsion (Figure 2). These results were used to help interpret the in-vivo microenvironments at the injection site, where the formulations were brought into contact with the extracellular fluids. Both the emulsion and the microemulsion formulations are unlikely to give a burst release of the low-pH drug solution following injection, given that there was a clear boundary between the formulation and the aqueous phase.

Short-term in-vitro cytotoxicity

HP-β-CD (0.1 M) alone and the vehicle components of the w/o emulsion did not cause significant cytotoxicity compared with the control (normal saline) in all three assays (Figure 3). Vehicle components of the microemulsion induced morphological changes in the L929 fibroblasts as shown in Figure 4 and showed some cytotoxicity in both MTS and LDH assays

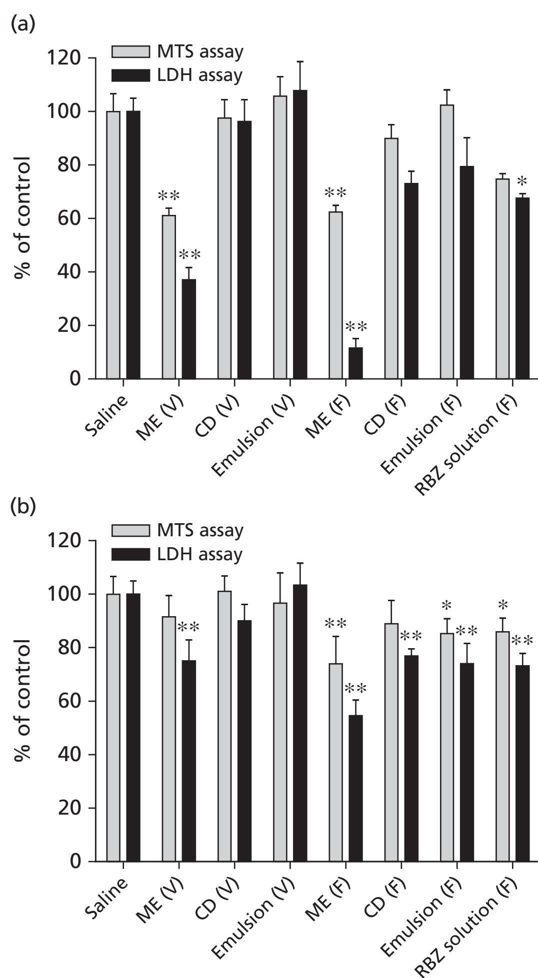

Figure 3 Cytotoxicity of ricobendazole formulations and vehicles to L929 fibroblast cells. Short-term (a) and long-term (b) cytotoxicity of RBZ 5% w/v formulations (F) and vehicles (V) to L929 fibroblast cells. Statistically significant cytotoxicity is indicated by $^*P < 0.05$; $^{**}P < 0.01$ or 0.001. Data are means ± SD. ($n = 6$). ME, microemulsion; CD, cyclodextrin; RBZ, ricobendazole.

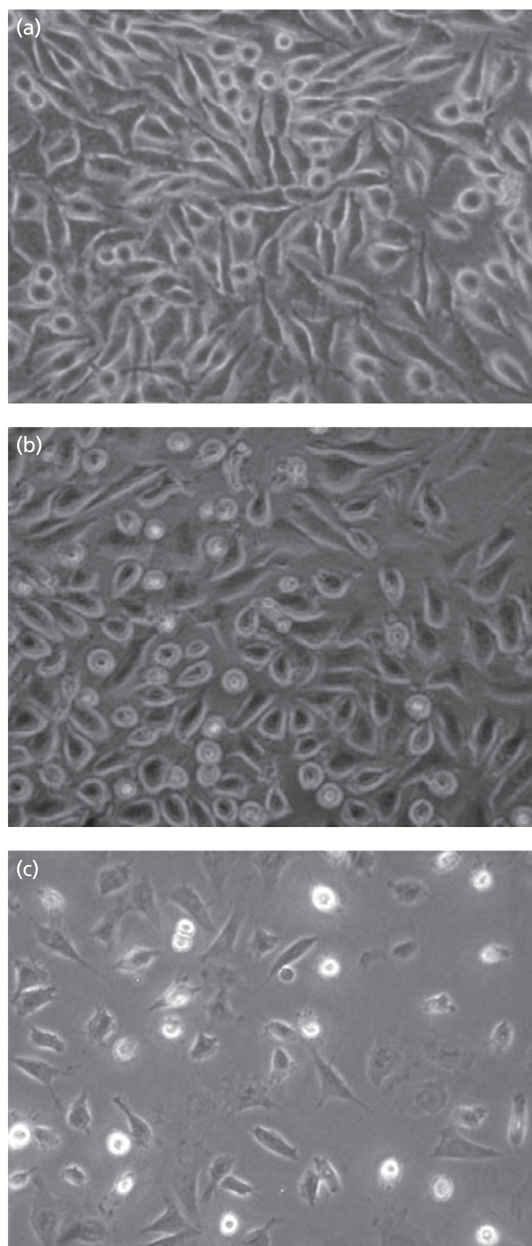

Figure 4 Light micrographs of L929 cells. (a), normal cells; (b), cells incubated with ricobendazole solutions showing mild cytotoxicity; (c), typical appearance of cells incubated with diluted microemulsion.

($P < 0.01$). In addition, HP-β-CD showed a protective effect to the cells against the RBZ (Figure 5).

Long-term in-vitro cytotoxicity

Longer-term cytotoxicity was evaluated at a higher formulation dilution (1 in 1000) to simulate an expected greater dilution at the site of injection with time. Minimal cytotoxicity was seen with the RBZ-containing cyclodextrin, emulsion and the reference formulations (Figure 3). No difference was observed between the emulsion or cyclodextrin-containing solution and drug solution alone ($P > 0.05$) in both MTS and LDH assays. However, the microemulsion

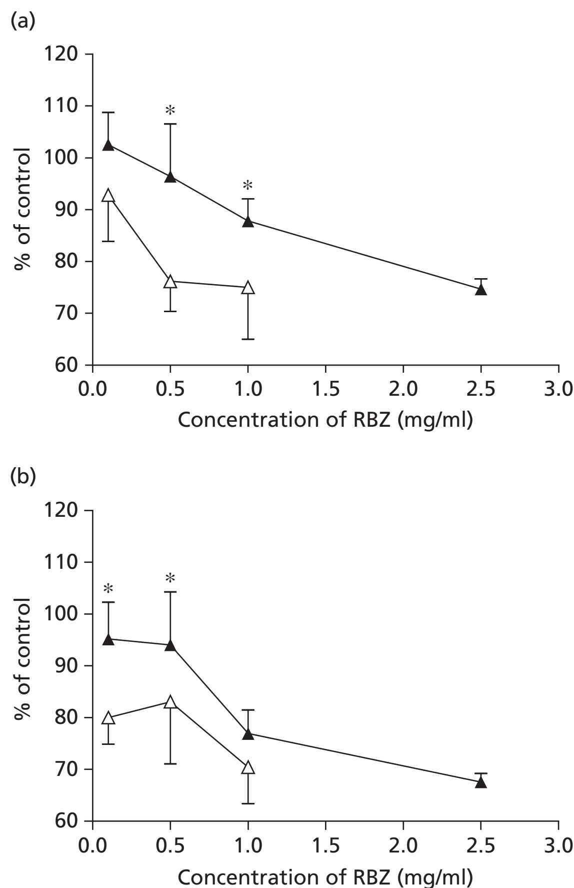

Figure 5 Cytotoxicity to L929 fibroblasts following 2 h exposure to ricobendazole. Cytotoxicity to L929 fibroblasts following 2 h exposure to RBZ (0–2.5 mg/ml) in the presence (molar ratio 1.2 : 1) (▲) and absence (△) of HP-β-CD, as determined by MTS (a) and LDH (b) assays. *Significant difference between two formulations ($P < 0.05$). Means ± SD ($n = 6$). RBZ, ricobendazole.

showed a greater cytotoxic effect ($P < 0.05$). Vehicle components of the w/o emulsion did not appear to cause significant cytotoxicity compared with the control (normal saline) in the MTS assay. A slight decrease in the intracellular LDH was seen with the HP-β-CD alone. The LDH assay showed that the microemulsion vehicle components had significant cytotoxicity ($P < 0.01$).

Tissue compatibility in sheep

Table 1 summarises the injection site reactions following s.c. injection of the formulations and the respective vehicles in sheep. Both the reference formulation and its vehicle (0.2 M HCl) caused rapid injection-site reactions. In contrast, injection of the three alternative vehicles (without drug) caused no visible injection-site reactions. The CD formulation gave transitory pain on injection. Plasma CK did not increase significantly (<300 IU/l) following injection of any of the formulations along with the respective vehicle, compared with injection of the normal saline control or the initial level (262 ± 80 IU/l). Microscopic observation of the tissue at the injection site, including epidermis, dermis, panniculus muscle and adipose tissue, obtained 2 weeks post-injection, showed that the reference formulation caused obvious tissue damage. However, the tissue damage was not observed at the injection sites that received other formulations or vehicles, including the HCl solution (Figure 6), with only minimal multifocal haemorrhage seen in the subcuticular fat tissue at 2 weeks post-injection of the CD formulation. Neither the formulation nor vehicle of the microemulsion showed any inflammation in the biopsy tissues.

Pharmacokinetics of ricobendazole following subcutaneous injection

After s.c. injection, the w/o emulsion produced a sustained drug-release profile, whereas the microemulsion gave similar concentrations to the control formulation (Figure 7). The CD formulation showed rapid absorption with a T_{max} of about 4 h, and a higher C_{max} compared to the low-pH reference formulation. The areas under the plasma concentration–time curve (AUC) for the three alternative formulations were all enhanced relative to the reference formulation (Table 2).

Discussion

Injectable formulations of poorly water-soluble drugs present a challenge, as solubilisation strategies are often associated with tissue irritation at the site of injection. Formulation factors reported to cause injection site reactions include: (i) the irritant nature of the drug or other formulation components, for instance, high content of co-solvent,[5] (ii) apparent pH of the final product (pH <3 or >9 for s.c. injection)[39] and (iii) post-injection precipitation of drug at the injection site. Formentini *et al* suggested that the tissue lesions following s.c. injection of an

Table 1 Injection site reactions post-injection of ricobendazole formulations and the vehicles

Site reactions		Normal saline	Reference	CD solution	Emulsion	Microemulsion
Pain on injection	F	–	+	Transient	–	±
	V	–	+	–	–	–
Appearance of swelling (diameter)	F	–	1.5–6 h (~6 cm)	–	–	–
	V	–	1.5–6 h (~6 cm)	–	–	–
Pain at palpation	F	–	+ First day	–	–	± First day
	V	–	+ First day	–	–	–
Maximum skin temperature, °C (tmax)	F	37.1 (25)	38.3 (225)	37.6 (38)	37.2 (129)	37.4 (91)
	V	37.3 (28)	38.3 (164)	37.3 (48)	37.3 (123)	37.5 (99)

Responses were graded as (–), no obvious reaction; (±), no significant reaction; (+), significant reaction. F, formulations; V, vehicles.

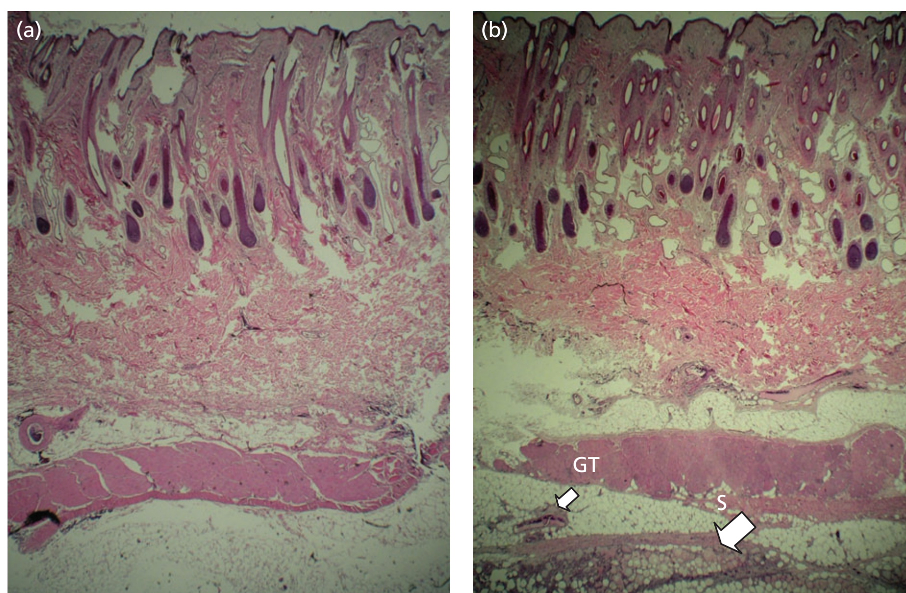

Figure 6 Histology of tissues at the injection sites 2 weeks after subcutaneous injection in sheep. (a), typical histology of normal saline or ricobendazole formulations and vehicles showing unremarkable changes; (b), reference formulation showing remarkable tissue damage. GT, granulation tissue; S, steatitis.

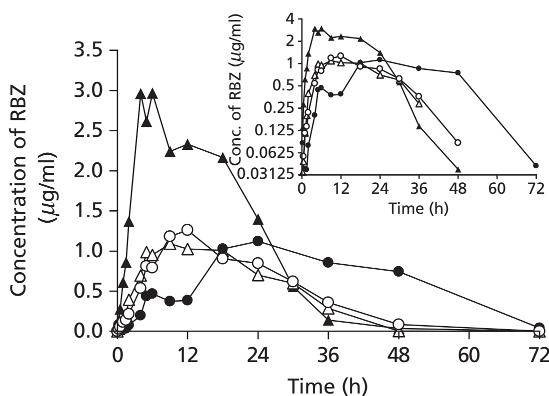

Figure 7 Plasma concentration–time profiles of ricobendazole in sheep after subcutaneous injection. △, reference formulation (aqueous low pH solution); ▲, HP-β-CD formulation; ○, microemulsion; ●, w/o emulsion. The insert was plotted in log-linear scale. RBZ, ricobendazole.

RBZ formulation were due to RBZ precipitation and slow re-dissolution at the injection site, rather than the low pH of the formulation.[33] In other words, reducing drug precipitation from the RBZ injectable is an important strategy for preventing tissue damage.

In the present study, three injectables were obtained by modification of an acidic RBZ solution: incorporation into a w/o emulsion, a microemulsion and addition of HP-β-CD. The reference formulation, an acidic RBZ solution without formulation modification, caused the greatest irritation on injection, with swelling and temperature elevation at the injection site and granulation tissue in the biopsy, suggesting significant tissue damage. This can be partially attributed to the low pH of the formulation. Once acid is released to the tissue it causes pain immediately, but it may be neutralised and/or absorbed rapidly before causing severe tissue damage. However, it was not just the local concentration of H^+ that caused the injection site reactions because the tissue responses to the vehicle (HCl solution) were not as severe as those of the formulation. The vehicle also caused pain and swelling but no noticeable histological changes in the biopsy sample taken at 2 weeks, which is consistent with the finding by Formentini *et al*.[33] The poor bioavailability of the reference formulation is suggested to be due to post-injection drug precipitation similar to that observed *in vitro*[35] and as reported for other pH solubilised drugs.[13,30]

The w/o emulsion, prepared using ethyl oleate as oil and Span 80 and PGPR as emulsifiers, showed good stability and syringability and was relatively easy to prepare. Ethyl oleate has been reported to be well tolerated by tissue after injection[40] and to be more rapidly absorbed than some vegetable oils,[41] possibly due to its low viscosity (3.9 mPa at 25°C[42]). Span 80 is a non-ionic ester of sorbitan and oleate, and has been approved by the Food and Drug Administration for intramuscular injection.[42] PGPR is a non-ionic surfactant and is a powerful water-in-oil emulsifier. It has not been approved for parenteral preparations but has been reported as non-toxic and pharmacologically inactive.[43]

The present cytotoxicity studies indicate that the materials used in emulsion formulation are non-toxic to L929 fibroblasts. The preliminary in-vitro studies showed that the formulation can control the burst release of irritant H^+ and therefore potentially create a barrier between tissue and the irritant low-pH solution. Microscopy observation of the buffer/emulsion interface suggested that no emulsion droplets rupture on dilution in an aqueous environment. This is thought to contribute to the mechanism of sustained drug and

Table 2 Pharmacokinetic parameters of ricobendazole formulations in the pilot study following subcutaneous injection to a single sheep

Pharmacokinetic term	Reference	CD solution	Emulsion	Microemulsion
C_{max} (μg/ml)	1.09	2.97	1.12	1.26
T_{max} (h)	9	4	24	12
k (h^{-1})	0.07	0.15	0.09	0.08
$t_{1/2}$ (h)	9.3	4.5	7.8	9.1
$AUC_{0-\infty}$ (μg h/ml)	29.0	56.5	45.6	32.3
Relative bioavailability (%)	100	194	155	111

CD, cyclodextrin.

acid release from the emulsion following s.c. administration. In sheep, the irritancy appeared to be successfully masked. In addition, the w/o emulsion showed a delayed absorption and the RBZ concentration remained low (0.4 μg/ml) over the first 12 h, then remained at above 1 μg/ml from about 18 to 48 h after injection. This is consistent with the drug-release rate being sustained by incorporation of the drug in an emulsion.[44,45] Additionally, if hydrogen ion release is also reduced, as suggested by the titration experiments (Figure 1), drug precipitation may have also been reduced. Overall, a sustained RBZ-absorption profile resulted, with increased AUC (155% compared to the reference).

So far, there has been no report in the literature on tissue tolerance to a microemulsion after s.c. administration. In the present study, the RBZ microemulsion formulation caused the greatest cellular toxicity to L929 fibroblasts. The vehicle alone was significantly cytotoxic, which is considered to be the result of the high content of surfactants in the formulation causing cellular damage by solubilisation of components in the membrane and subsequently leakage of the intracellular enzymes such as LDH.[46] Despite the high in-vitro cytotoxicity of the microemulsion, neither the formulation nor the vehicle alone appeared to cause injection site reactions in sheep. Microscopy studies suggested the microemulsion did not readily disperse in aqueous media. Rather, a layer of coarse emulsion formed at the interface. *In vivo*, this emulsion layer may act as a barrier to prevent a burst release of the drug and other components of the formulation, for example H^3O^+ and the surfactants. Another study suggested that incorporation of oil in a surfactant system could decrease the toxicity to cell membranes, due to the formation of surfactant–oil aggregates, and consequently the solubilising capacity for the cell membrane components would be reduced.[47] The plasma–time profiles of the microemulsion and the reference formulation were similar except for a slight delay in t_{max} for the microemulsion (12 h vs 9 h).

Cyclodextrins have been reported to remove cholesterol from cell membranes. However, HP-β-CD has been reported to be well-tolerated as an injectable drug carrier *in vitro* and *in vivo*.[23,25] In this study, a 20% HP-β-CD solution following s.c. injection did not cause tissue irritation. HP-β-CD had some protective effect against cytotoxicity of RBZ in the cell culture model. Following s.c. administration, the AUC and C_{max} were about two and three times higher than those of the low-pH RBZ solution, suggesting that HP-β-CD significantly increased the bioavailability of RBZ. The terminal rate constant (k) 0.15 h^{-1}, was greater than the other three formulations (0.07–0.09 h^{-1}) but similar to that reported for intravenous administration in sheep (0.14 h^{-1}),[48] supporting the assumption that absorption was complete for the HP-β-CD formulation. This is postulated to be due to the inhibitory effect of HP-β-CD on RBZ precipitation at the injection site.[35] If the bioavailability of the drug could be improved, a smaller dosage of RBZ may be equally effective and a formulation with lower RBZ concentration may further reduce the irritancy. Further studies have been conducted to investigate the reproducibility of the in-vivo response for the HP-β-CD formulation and will be reported.

Cell culture models have been used as an in-vitro tool to predict local cellular damage.[10,23,47,49] In the present study, cytotoxicity was assessed by MTS and LDH leakage assays. By using multiple assays, the cytotoxicity mechanisms may be differentiated. The MTS assay reflects the detrimental intracellular effects of test chemicals on mitochondria and metabolic activity (e.g. cell viability). Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme, present in all cell types, that is released rapidly on cell death or membrane damage. Except for the emulsion vehicle components, all other formulations and vehicles showed higher cytotoxicity in the LDH assay than in the MTS assay, suggesting enzyme leakage in the surviving cells due to cellular membrane damage. Overall, the results suggest that the diluted vehicle components of the emulsion and cyclodextrin formulations were of low toxicity in contrast to the microemulsion. However, this study found no significant in-vitro/in-vivo irritation correlation with respect to cytotoxicity and injection site irritancy. It appeared that minimised irritation reactions correlated with the potential to control release of H^+ from the formulations, rather than the cytotoxicity. In addition, the cell culture model cannot predict irritation effects caused by low pH or drug precipitation at the injection site, but ranked the cellular toxicity of soluble components. Furthermore, in-vitro models have no vasculature, innervations or inflammatory cells, and although attempts were made at least to mimic formulation dilution at the site of injection, these effects may be greater *in vivo*.

Conclusions

This single animal study suggests that all three formulations containing the low-pH RBZ solution had improved tissue tolerance and increased bioavailability compared with the low-pH RBZ solution (reference formulation). Injection site reactions may be minimised by identification of irritant components in a formulation and by modulating their release.

Controlling the burst release of the poorly water soluble drug RBZ in a low-pH solution may improve tissue tolerance and minimise post-injection precipitation, and hence increase drug bioavailability. The study also suggests that HP-β-CD is a useful local injectable carrier to enhance the absorption of RBZ after s.c. injection in sheep. The w/o emulsion offers an appealing alternative approach for delivering poorly soluble drugs solubilised at low pH, due to the sustained release of H^+ and the drug, leading to an injectable that is well tolerated by tissue and with improved bioavailability. The in-vitro cytotoxicity studies did not predict in-vivo irritation effects in this study.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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