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Factors limiting the oral bioavailability of *N*-acetylglucosaminyl-*N*-acetylmuramyl dipeptide (GMDP) and enhancement of absorption in rats by delivery in a water-in-oil microemulsion

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Abstract

The bioavailability (BA) of radio-labelled *N*-acetylglucosaminyl-*N*-acetylmuramyl dipeptide (GMDP) was low when administered by oral gavage as an aqueous solution to conscious male Sprague–Dawley rats ($8.3 \pm 4.4\%$ (mean $\pm$ S.D., $n = 3$)). To assess the likely factors contributing to the poor BA of GMDP, the stability of GMDP in the lumen of the gastrointestinal (GI) tract was examined in vitro, using ex vivo GI contents. GMDP was degraded by the contents of the small intestine, caecum and large intestine but was more stable in stomach contents. The permeability coefficient (p_{app}) of GMDP in isolated sections of rabbit ileum was 1.67×10^{-6} cm/s in the mucosal to serosal direction and was not significantly different in the serosal to mucosal direction, indicating that GMDP is poorly permeable and passively transported across the intestinal wall. First pass metabolism was considered to be unlikely to be the primary limitation to the oral bioavailability of GMDP and therefore, that the oral bioavailability of GMDP was likely limited by instability in the lumen of the gastrointestinal tract and low intestinal permeability. A water-in-oil (w/o) microemulsion formulation subsequently developed to address these problems was trialed in a preliminary bioavailability study in rats and enhanced the bioavailability of GMDP ten-fold when administered intraduodenally, indicating that w/o microemulsions may represent a viable mechanism for enhancing the bioavailability of poorly GI-stable and poorly permeable peptide-based molecules. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Microemulsion; GMDP; Intestinal instability; Intestinal permeability; Peptide; Absorption

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1. Introduction

N-acetylglucosaminyl-*N*-acetylmuramyl dipeptide (GMDP) is an *N*-acetylglucosamine analogue of muramyl dipeptide (MDP). MDP is the smallest fragment of the bacterial cell wall which initiates an immune response in man (Ellouz et al., 1974). MDP and MDP analogues have been identified as having potential clinical utility as vaccine adjuvants (Ellouz et al., 1974), immunostimulators (Tsubura et al., 1988), anti-cancer agents (Utsugi et al., 1991) and antiinflammatory agents (Leclerc et al., 1979) however, MDP is highly pyrogenic in man (Dinarello et al., 1978). GMDP retains the favourable activity of MDP but is less pyrogenic.

Oral administration is desirable for most therapeutic agents, including peptides and proteins, as it avoids the pain and risk of infection associated with parenteral administration and thereby leads to greater patient compliance. The development of peptides and proteins as oral formulations has however, been limited by their low bioavailability when administered via this route. In this regard, initial studies examining the oral bioavailability of (^{14}C)GMDP in beagle dogs have indicated that the absolute bioavailability of GMDP is low (6.7%, unpublished data), however, limited information is available regarding the factors contributing to the poor oral bioavailability.

The low oral bioavailability of GMDP was confirmed in the current study in the rat. The stability of GMDP in the lumen of the gastrointestinal tract and intestinal permeability were subsequently examined and the data reported here lead to the conclusion that the oral bioavailability of GMDP is limited primarily by instability in the lumen of the gastrointestinal tract and low intestinal permeability. The dissolution of GMDP in an aqueous environment is unlikely to contribute to the low oral bioavailability as the peptide is highly water-soluble (1 g of peptide is dissolved in 0.9 ml of water). Consequently formulation strategies which protect the compound from breakdown in the gut and increase the intestinal permeability will likely enhance the oral bioavailability of GMDP.

In the light of these findings, a water-in-oil microemulsion system similar to that previously reported to enhance the oral bioavailability of peptides in rats (Constantinides et al., 1994; Constantinides, 1995; Constantinides et al., 1996) was selected as a prototype delivery system with which to conduct a preliminary bioavailability study. The data described indicate that water-in-oil microemulsion systems may improve the delivery characteristics of peptides when enzymatic/chemical degradation in the lumen of the gut and limited intestinal permeability are the cause of reduced oral bioavailability.

2. Materials and methods

2.1. Materials

Three batches of [^{14}C]GMDP (specific activity of 175 μCi/μmole, 194 μCi/μmole and 182 μCi/μmole and one batch of (^{3}H)GMDP (specific activity 1.27 Ci/mmole) were custom synthesised by Auspep Pty. (Parkville, Vic., Australia). Peptech (North Ryde, NSW, Australia) supplied the GMDP. (^{14}C)mannitol (Cat No. NEC 314), (^{3}H)mannitol (Cat No. NET 101) and (^{14}C]diazepam (Cat No. NET 564) were obtained from NEN (Boston, MA). Captex 355 and Capmul MCM were obtained from Abitec (Janesville, WI). Tween 80 (Polyoxyethylene-sorbitan monooleate) was obtained from Sigma Chemical (St Louis, MO). All other reagents were at least AR grade.

2.2. HPLC analysis of GMDP

HPLC analysis of samples for parent (^{14}C)GMDP or (^{3}H)GMDP was carried out using a Waters HPLC system consisting of a 600E System Controller and gradient pump, 486 tuneable absorbance detector, 712 WISP autosampler and Maxima software. A Pharmacia Frac-100 fraction collector was used to collect the eluant from the HPLC system for analysis by liquid scintillation counting.

Samples were analysed for (^{14}C)GMDP or (^{3}H)GMDP content by isocratic, reverse phase

HPLC. (10% MeOH/90% H_2O, Phenomenex Columbus 5 μm C18 250 × 4.6 mm column, 100 μl injection volume, 1 ml/min). Samples were injected (100 μl) and eluant from the column was collected for 20 min (1 ml aliquots) using a Frac 100 Fraction Collector (Pharmacia). These fractions were counted for ^{14}C or ^{3}H by liquid scintillation counting. The two GMDP anomers were contained in separate peaks on the chromatogram (8–11 and 14–17 min, respectively). The radioactivity associated with these peaks was combined to give total radio-labelled GMDP in plasma. A standard curve consisting of plasma samples spiked with a known quantity of (^{14}C)GMDP or (^{3}H)GMDP was used to determine the concentration (ng equivalents/ml) of GMDP in each plasma sample. The limit of quantitation for both (^{14}C)GMDP and (^{3}H)GMDP was 1 ng/ml. Reproducibility, expressed as % CV, varied by $< 10\%$ and accuracy was within $\pm 10\%$ of theoretical. Standard curves were linear over the range 1–500 ng/ml for (^{14}C)GMDP and 1–200 ng/ml for (^{14}H)GMDP.

2.3. Liquid scintillation counting

^{14}C and ^{3}H activity in samples was determined by liquid scintillation counting using StarScint Scintillation Cocktail (Packard, Meriden, CT) and a Packard Tri-Carb 2000CA Liquid Scintillation Analyser.

2.4. In vitro stability studies

The contents of the stomach, small intestine, caecum and large intestine were isolated from overnight fasted male Sprague–Dawley rats (250–300 g) under anaesthesia with 2% isofluorane. The contents of each section of the GI tract were placed in separate plastic screw capped tubes with 4 ml of Ringer's solution to create a slurry. Each tube was spiked with (^{14}C)GMDP (8 μCi (32 μg) in a volume of 20 μL of H_2O and unlabelled GMDP (66 μl of 1 mg/ml in H_2O) was included to increase the mass of GMDP in each tube to a more practical concentration (total of 98 μg GMDP ($\approx 60\%$ of the oral dose given in the BA study)). A tube containing 4 ml of Ringer's solution was also spiked with GMDP and run as a control. A zero time aliquot (0.5 ml) was then taken from each mixture, centrifuged and the supernatant immediately frozen for later analysis. The remaining mixtures were incubated at 37°C and 0.5 ml aliquots were removed each h for 4 h and treated in the same way as the sample taken at 0 h. The experiment was repeated using the luminal contents from three different rats.

To process samples for HPLC analysis of parent (^{14}C)GMDP, chloroform (500 μl) and 10% trichloroacetic acid (TCA) (100 μl) were added to each aliquot. Samples were vortexed and centrifuged at 2600 × *g* for 5 min. The aqueous top layer was removed from each tube and filtered by centrifugation at 2600 × *g* for 5 min in 0.22 μm cellulose acetate microcentrifuge filters (8000 Series Microcentrifuge Filters, Lida, Kenosha, WI). HPLC was used to determine the content of intact parent compound at each time point. The extent of (^{14}C)GMDP instability was assessed by comparison of peak areas for GMDP at later time points relative to the profile obtained at time 0 (no instability).

2.5. In vitro intestinal permeability studies

The methods employed are modified from experimental procedures well described in the literature (Smith, 1996). New Zealand white male rabbits were killed by over-dose with pentobarbitone administered by intravenous injection. The distal part of the ileum, starting 5 cm proximal to the ileo-caecal junction was isolated. The serosa and muscle layers were removed and the tissue was opened along the mesenteric border. Mucus and lumen contents were removed by washing the tissue with cold Ringer's solution. Segments of tissue were mounted in modified Ussing chambers (Navicyte, Sparks, NY). Care was taken to avoid including Peyer's patches in the tissue mounted in each chamber. The chambers were filled with bicarbonate buffer solution (pH 7.4 when gassed with O_2/CO_2 (5%/95%)). Glucose (10 mM) was included in the buffer solution on the serosal side of the tissue as an energy source. Mannitol (10 mM) was included in the buffer on the mucosal side to compensate for any alteration in osmotic

pressure caused by the addition of glucose to the serosal buffer. Chambers were gassed continuously with O_2/CO_2, (5%/95%) and heated to 37°C. The tissue was allowed to equilibrate for 30 min during which time the buffer solutions were changed twice. After the equilibration period radio-labelled marker compounds ((^{14}C)mannitol and (^{3}H)diazepam) or GMDP were introduced into the chambers on one side of the tissue (donor chamber). The movement of the radio-labelled compounds from the donor chamber to the receiver chamber of the diffusion cell was subsequently quantified. Samples (100 μl) were taken from the receiver chamber at 20 min intervals for 2 h and liquid scintillation counted for ^{3}H and/or ^{14}C content as appropriate. The sample volume removed was replaced with fresh buffer solution following each sampling.

The integrity of the GMDP in the mucosal and serosal chambers at the completion of sampling was confirmed by HPLC. Samples (200 μl) from the donor and receiver chambers were filtered through 0.22 μm cellulose acetate membranes filters (8000 Series Microcentrifuge Filters, Lida, Kenosha, WL) to remove any particulate material and analysed by HPLC.

The apparent permeability coefficient (P_{app} (cm/s)) was calculated by the following relationship:

$$P_{app} = \frac{V\left(\frac{dC}{dt}\right)}{A \cdot C}$$

Where V is the volume (ml) of the receiver cell, A is the surface area (cm^2) of the exposed tissue, C is the initial donor concentration and dC/dt is the change in the concentration of the compound in the receiver cell over time.

2.6. Preparation of the microemulsion formulation

Pseudo-ternary phase diagrams were constructed to examine the formation of w/o microemulsions using a four component oil/surfactant/aqueous phase system. The four component system consisted of:

1. A medium-chain fatty acid based triglyceride (Captex 355).
2. A low hydrophile-lipophile balance (HLB) surfactant (Capmul MCM).
3. A high HLB surfactant (tween 80).
4. Milli Q water (aqueous phase).

The ternary plots were constructed by keeping the ratio of Captex 355 and Capmul MCM constant and varying the remaining two components. Regions of the phase diagram in which w/o microemulsions exist were determined by titration with the aqueous phase (water). Captex 355/Capmul MCM were held in a fixed ratio of 4:1 and titrated against increasing amounts of tween 80 and water. Points of phase separation, turbidity and transparency were noted. Regions in which the formulation formed visually stable, transparent solutions were marked on the phase diagram as a microemulsion. The final formulation identified for use in subsequent animal studies contained 65% Captex 355, 22% Capmul MCM, 10% tween 80 and 3% aqueous phase (containing GMDP).

The water-in-oil microemulsion formulation was prepared by sequentially adding and mixing the four components of the microemulsion system. Captex 355 and Capmul MCM were combined in a ratio of 4:1 by weight and then gently mixed on a roller mixer. Tween 80 was added by weight to the Captex/Capmul solution to achieve a final concentration of 10% w/w in the formulation (87% Captex/Capmul: 10% tween 80: 3% water). In the microemulsion studies logistical details dictated that (^{3}H)GMDP and not (^{14}C)GMDP be used as a tracer. (Subsequent intravenous studies of the pharmacokinetics of (^{3}H)GMDP demonstrated that there was no significant difference between (^{3}H)GMDP and (^{14}C)GMDP pharmacokinetics). (^{3}H)GMDP (20 μL (≈ 80 μCi of stock solution supplied from Peptech (GMDP dissolved in 0.1 M HCl)) and unlabelled GMDP (10 μL of 10.4 mg/ml solution of GMDP in water) were added to the formulation in the aqueous phase and mixed gently on a roller mixer.

The total ^{3}H activity/g of the formulation was determined by liquid scintillation counting. Three aliquots (≈ 5 μl) of the formulation were accurately weighed into 6 ml plastic counting vials (Packard) and 3 ml, of Star-Scint™ (Packard) was added and vortexed.

2.7. Bioavailability studies

Male Sprague–Dawley rats (250–300 g) were anaesthetised with 2% isofluorane to facilitate surgery to implant cannulas (polyethylene tube, i.d. 0.58 mm, o.d. 0.96 mm; PE-50, Clay Adams, USA) into the left jugular vein, left carotid artery and duodenum as required by the individual study. Cannulas were tunnelled subcutaneously to exit at the back of the neck and connected to a swivel/leash assembly to facilitate unrestricted access to the jugular vein (for IV dosing), carotid artery (for sampling of systemic blood) and duodenum (for intra-duodenal dosing). Animals were placed in metabolism cages to facilitate collection of urine and faeces and allowed to recover overnight prior to dosing. (^{14}C)GMDP is, in part, excreted in CO_2, via the lungs, therefore cages were placed in a fume cupboard. To offset potential cooling due to drafts in the fume cupboard, supplementary heating was provided with ceramic bulb heaters.

2.7.1. Intravenous administration of (^{14}C)GMDP

Rats were dosed by intravenous infusion via the jugular cannula over 1 min with 0.056 mg/kg (^{14}C)GMDP (3.5 μCi/rat) in 1 ml of normal saline. Blood samples (400 μl) were taken via the carotid carmula at time 0 (end of infusion), 5, 15, 30, 60 and 90 min and 2, 2.5 and 3 h. The plasma was isolated by centrifugation at 2600*g* for 5 min and the samples were stored at −20°C for later analysis of total activity and parent compound.

2.7.1.1. Pharmacokinetic calculations. Distribution of (^{14}C)GMDP was rapid and elimination of (^{14}C)GMDP exhibited one compartment pharmacokinetics. The elimination rate constant (k) of parent drug was calculated using linear regression over the period 0–2.5 h and the half-life ($t_{1/2}$) of GMDP was calculated from $\ln 2/k$. The area under the plasma concentration-time curve ($AUC^{0-\infty}$) was calculated using the linear trapezoidal rule to 2.5 h, followed by addition of the extrapolated area ($AUC^{2.5-\infty}$) calculated by dividing the measured concentration at 2.5 h by the elimination rate constant (k). Less than 5% of the total AUC for parent (^{14}C)GMDP was contained in the extrapolated area. The systemic clearance of parent drug was calculated from dose/$AUC^{0-\infty}$.

2.7.2. Oral administration of GMDP in aqueous solution

Rats were anaesthetised briefly with isofluorane to allow oral dosing. (^{14}C)GMDP (0.45 mg/kg (28.5 μCi/rat)) was administered orally by gavage in 1 ml of H_2O. The dose was then rinsed in with an additional 1 ml of H_20. Blood samples (400 μL) were taken via a carotid cannula at 0, 15, 30, 45 min and 1, 1.5, 2, 2.5, 3, 4 and 6 h to facilitate the determination of plasma levels of both total ^{14}C activity and (^{14}C)GMDR Further samples were taken at 8, 10 and 12 h and every 12 h after this point up to 72 h, however, smaller samples (200 μl) were taken to allow the determination of total ^{14}C in plasma only. The plasma was isolated from blood samples by centrifugation at $2600 \times g$ for 5 min.

The metabolic cages also allowed for the collection of urine and faecal material during the course of the experiment. Urine was collected at intervals of 0–12, 12–24, 24–48 and 48–72 h while faeces excreted by each rat over 24 h periods during the experiment was collected and pooled. These samples were stored at −20°C before analysis.

2.7.2.1. Pharmacokinetic calculations. The elimination rate constant (k) of parent drug was calculated using linear regression over the terminal elimination phase and the half-life ($t_{1/2}$) was calculated from $\ln 2/k$. The area under the plasma concentration-time curve ($AUC^{0-\infty}$) was calculated using the linear trapezoidal rule to 6 h, followed by addition of the extrapolated area ($AUC5^{6-\infty}$) calculated by dividing the measured concentration at 6 h by the elimination rate constant (k). Less than 5% of the total AUC was contained in the extrapolated area. The absolute oral BA of GMDP was calculated by comparison of the dose corrected $AUC^{0-\infty}$ after oral and intravenous administration of (^{14}C)GMDP and expressed as a percentage.

2.7.2.2. Intra-duodenal administration of GMDP in a water-in-oil microemulsion. A 1 ml dose of

the water-in-oil microemulsion containing 128.3 μg/g GMDP (0.45 mg/kg) was delivered as a bolus over 1 min via an intraduodenal cannula and the cannula was cleared with 0.2 ml of normal saline. The microemulsion formulation dose of GMDP (128.3 μg/g of formulation, ≈0.5 mg/kg animal) consisted of 104.6 μg/g of unlabelled GMDP and 23.7 μg/g (43 μCi) of parent (^{3}H)GMDP. Blood samples (400 μl) were taken at 0, 15, 30, 45 min and 1, 1.5, 2, 2.5, 3 4 and 6 h. The plasma was isolated from samples by centrifugation at 2600*g* for 5 min and stored at −20°C for analysis. Urine was collected from the metabolic cages at intervals of 0–12, 12–24, 24–48, 48–72 and 72–120 h. Faecal material was also collected at 24 h intervals during the course of the experiment. These samples were stored at −20°C before analysis for total ^{3}H activity and parent (^{3}H)GMDP in urine samples.

2.7.2.3. Pharmacokinetic calculations. The elimination rate constant (k) of parent drug was calculated using linear regression and the half-life ($t_{1/2}$) was calculated from $\ln 2/k$. The area under the plasma concentration-time curve ($AUC^{0-\infty}$) was calculated using the linear trapezoidal rule to 6 h, followed by addition of the extrapolated area ($AUC^{6-\infty}$) calculated by dividing the measured concentration at 6 h by the elimination rate constant (k). Less than 5% of the total AUC was contained in the extrapolated area. The BA of GMDP was calculated by comparison of the dose corrected $AUC^{0-\infty}$ after intraduodenal and intravenous administration of GMDP and expressed as a percentage.

2.8. Processing of samples for determination of total activity and/or parent GMDP in plasma, urine and faeces

2.8.1. Plasma

Plasma was isolated from blood samples by centrifugation at $2600 \times g$ for 5 min. A 20 μl aliquot of the plasma sample was then added to 3 ml Star-Scint™ (Packard) liquid scintillation cocktail and the samples were vortexed and counted for ^{14}C or ^{3}H content. An aliquot of 200 μl was taken from the remaining plasma and TCA added (40 μl, 10% w/v aqueous solution) to precipitate plasma proteins. The samples were mixed and 200 μl of chloroform was added to each sample. Samples were vortexed and centrifuged at $2600 \times g$ for 5 min. The aqueous layer (top) was removed and assayed for parent [^{14}C]GMDP or (^{3}H)GMDP by HPLC.

2.9. Urine

The base of the metabolic cage was rinsed with Milli Q water at the completion of each time period to facilitate the collection of all ^{14}C or ^{3}H labelled material excreted in the urine. All of this material (urine plus washings) was collected as a single sample and stored at −20°C. A 200 μl aliquot of each urine sample was analysed by HPLC to determine the percentage of ^{3}H or ^{14}C label associated with parent GMDP. The remaining volume was divided into 3 ml samples in 20 ml plastic liquid scintillation counting vials and 15 ml of Star-Scint™ (Packard) was added. Samples were liquid scintillation counted for ^{14}C or ^{3}H activity. The results of these samples were pooled to give a total amount of activity excreted in the urine over the sampling period. The percentage of total activity excreted in the urine that could be attributed to parent drug was then calculated.

2.9.1. Faeces

Faeces was collected and pooled at 24 h intervals from metabolic cages during the course of the pharmacokinetic studies. Milli Q water was added to each faeces sample to create a slurry which was dried at 80°C. The dry weight of each sample was determined and ≈20 mg of each sample was then weighed accurately. Samples were rehydrated with water (0.2 ml) over 30 min after which Soluene-350™ (Packard) (2 ml) was added and the samples were incubated at 50°C until the faeces was solubilised. Isopropanol (1 ml) was added and the samples were incubated at 50°C for a further 2 h. Hydrogen peroxide (0.4 ml, 30% w/v) was then added dropwise with swirling and the samples were left to stand at RT until all bubbling had stopped. Star-Scint™ (Packard) was then added (15 ml) and ^{14}C activity was determined by liquid scintillation count-

ing. The total ^{14}C or ^{3}H activity in each faeces sample was then calculated using the dry weight of the total sample.

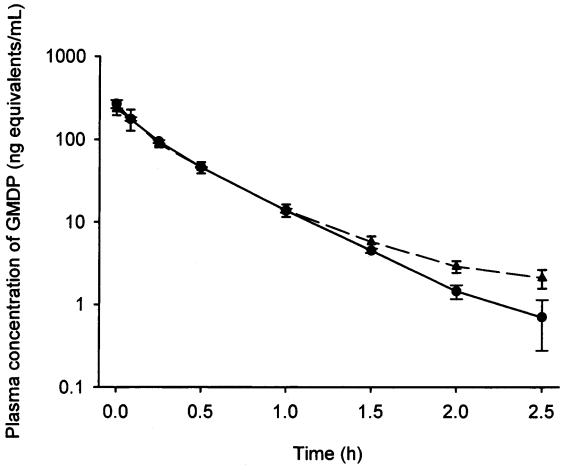

Fig. 1. Plasma concentration (mean ± S.D., $n = 4$) of total (^{14}C) activity in plasma (▲) and parent (^{14}C)GMDP (●) following intravenous administration of (^{14}C)GMDP (0.056 mg/kg) to rats.

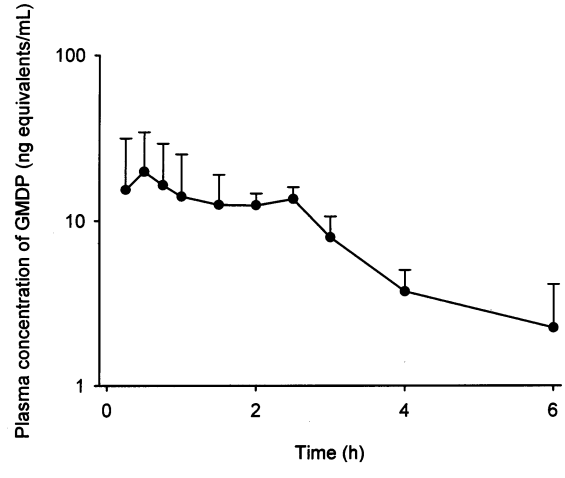

Fig. 2. Plasma concentration (mean ± S.D. $n = 3$) of parent (^{14}C)GMDP following oral administration of an aqueous solution of (^{14} C)GMDP (0.45 mg/kg) to rats.

3. Results

3.1. Pharmacokinetics of GMDP following intravenous administration

The plasma concentration-time profile for parent (^{14}C)GMDP after i.v. administration (mean ± S.D., $n = 3$) is shown in Fig. 1. Total ^{14}C activity is also shown and expressed as ng GMDP equivalents. Total ^{14}C activity may also represent activity associated with species other than [^{14}C]GMDP and therefore, total ^{14}C activity in ng GMDP equivalents will only accurately express GMDP concentration if the only labelled species present is GMDR Limited deviation of the total ^{14}C activity trace from the plasma profile of parent [^{14}C]GMDP suggest that the extent of metabolism of GMDP after i.v. administration is limited. The elimination half-life of (^{14}C)GMDP in the rat, calculated between 0 and 2.5 h was 0.26 ± 0.002 h^{-1} and the systemic clearance was 681 ± 105 ml/h/kg. Analysis of urine samples collected over the 8 h sampling period of the intravenous study indicated that at least 60% of the total activity dosed was excreted renally. HPLC analysis of urine revealed that 82% of the ^{14}C activity in the urine was associated with parent GMDP.

3.2. Oral bioavailability of GMDP

Fig. 2 shows the plasma concentration–time profile for (^{14}C)GMDP up to 6 h after administration of 0.4 mg/kg (^{14}C)GMDP by gavage as an aqueous solution. The concentration of (^{14}C)GMDP in plasma samples taken after 6 h was below the level of quantitation (1 ng/ml). (^{14}C)GMDP reached a maximum concentration of 19.65 ± 13.72 ng equivalents/ml (mean ± S.D., $n =$ three rats) 30 min after dosing and an oral bioavailability of 8.3 ± 4.4% was calculated for parent (^{14}C)GMDP. The total ^{14}C activity in plasma following the same oral dose is shown in Fig. 3 (mean ± S.D., $n =$ three rats). In contrast to parent GMDP, the total activity levels in plasma were much higher and reached a maximum much later (8 h) (Fig. 3). Analysis of urine samples collected at 12, 24, 48 and 72 h post oral dose indicated that a total of 5.5 ± 5.0% of the

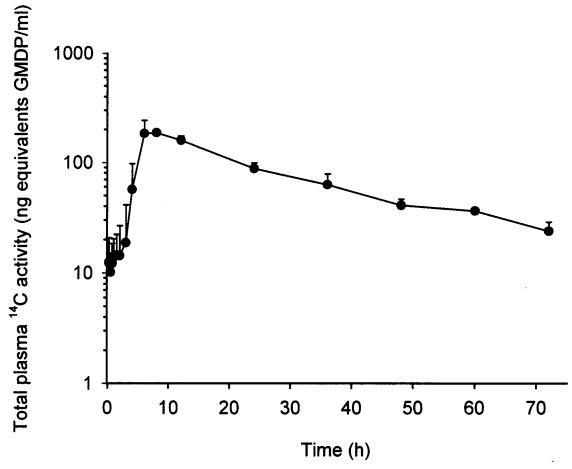

Fig. 3. Plasma concentration (mean ± S.D., $n = 3$) of total ^{14}C activity in plasma following oral administration of (^{14}C)GMDP (0.45 mg/kg) in aqueous solution.

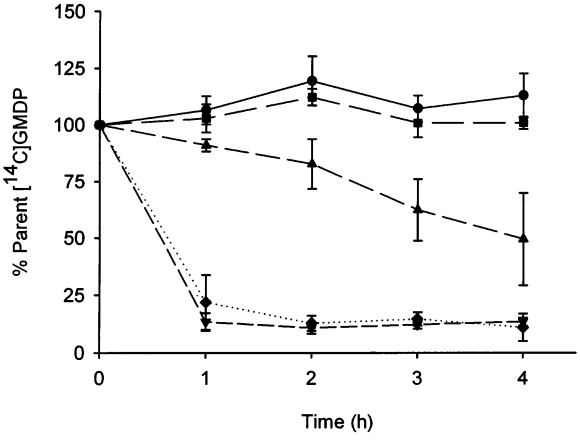

Fig. 4. The percentage of [^{14}C]GMDP remaining intact after incubation in vitro at 37°C with the lumen contents isolated from sections of rat gastrointestinal tract. Symbols represent incubations containing ringers solution (●), stomach contents (■), small intestine contents (▲), caecum contents (▼) and large intestine contents (black diamond). The data are presented as mean ± S.D. for n = three rats.

administered GMDP was eliminated intact in the urine up to 72 h. Faecal material excreted over the course of the experiment was also analysed for total ^{14}C activity by liquid scintillation counting and a total of 20.4 + 6.9% (mean ± S.D., n = three rats) of the oral dose was accounted for in the faeces up to 72 h. The majority of the ^{14}C activity excreted in the faeces (14.1 ± 5.8% of the dose) was accounted for in the 0–24 h sample. Smaller amounts of ^{14}C activity were excreted in the subsequent 24 h periods.

3.3. In vitro determination of the luminal stability of GMDP

(^{14}C)GMDP was degraded to varying extents by ex-vivo luminal contents of the small intestine, caecum and large intestine in vitro over the 4 h period tested (Fig. 4). The breakdown of (^{14}C)GMDP was more extensive and occurred more rapidly in the contents from the caecum and large intestine with 13.4 ± 3.9% and 21.9 ± 11.9% (mean ± S.D., $n = 3$) remaining intact after 1 h, respectively. GMDP was stable in the stomach contents showing no mean breakdown, while in small intestinal contents 49.6 ± 20.3% of the labelled GMDP remained intact after 4 h.

3.4. In vitro determination of intestinal permeability of GMDP

The intestinal permeability coefficients of (^{14}C)GMDP and the paracellular marker (^{14}C)mannitol and transcellular marker (^{3}H)diazepam were determined in sections of isolated rabbit ileum in vitro in modified Ussing chambers. The intestinal permeability of (^{14}C)GMDP was low and lower than the poorly permeable and poorly absorbed hydrophilic marker mannitol. The mean permeability coefficients obtained for each of the compounds are given in Table 1. The P_{app} values obtained in the mucosal to serosal direction were not significantly different to the values obtained in the reverse (serosal to mucosal) direction (data not shown), indicating that GMDP and the model marker compounds mannitol and diazepani were passively transported across the intestinal wall.

3.5. Bioavailability of GMDP following intraduodenal administration in a water-in-oil microemulsion

The concentration of GMDP in the plasma following intraduodenal administration of GMDP

Table 1
Permeability coefficients determined using isolated rabbit ileum in Ussing cells[a]

Compound	Direction	Permeability coefficient (cm/s) (mean ± S.D.)	*n*
GMDP	M → S	$1.67\ (\pm 0.04) \times 10^{-6}$	18 from three rabbits
Mannitol	M → S	$2.82\ (\pm 0.08) \times 10^{-6}$	18 from three rabbits
Diazepam	M → S	$1.18\ (\pm 0.03) \times 10^{-5}$	36 from six rabbits

[a] Direction refers to the movement of the radio-labelled compound from the mucosal (M) to serosal (S) side of the tissue.

(128 μg/g GMDP (23.7 μg/g (^{3}H)GMDP and 104.6 μg/g unlabelled GMDP)) contained in a w/o microemulsion is shown in Fig. 5 (mean ± S.D., $n = 4$ rats). The pharmacokinetic parameters calculated for GMDP after oral and intraduodenal administration are given in Table 2. Administration in the w/o microemulsion led to a large (ten-fold) increase in the bioavailability to 80.1 ± 30.4%

4. Discussion

The low absolute oral bioavailability of (^{14}C)GMDP (8.3 ± 4.4%) found in the current study confirms previous unpublished studies for GMDP in beagle dogs (oral bioavailability 6.7%, unpublished data, Peptech, Sydney) and is consistent with the low oral bioavailability of many peptide-like drugs. The oral bioavailability of peptide-like drugs may be limited by poor dissolution, breakdown in the gastrointestinal (GI) tract prior to absorption, significant first pass metabolism or limited permeability across the GI mucosa (Sarclaux et al., 1995; Langguth et al., 1997). GMDP is highly water-soluble (aqueous solubility > 1 g/ml) and dissolution is therefore, unlikely to represent a significant barrier to absorption.

Total ^{14}C activity in the plasma was high up to 120 h after dosing, suggesting that GMDP underwent significant post-absorptive metabolism either within the gut wall or in the liver, or was degraded by enzymes present in the lumen of the gastrointestinal tract or in the enterocyte brush border, prior to absorption of ^{14}C labelled GMDP breakdown products. The large differences in plasma levels of the parent drug and total activity and the lack of significant systemic metabolism of GMDP, suggested that luminal instability was a more likely explanation. In addition, luminal instability has previously been shown to limit the oral bioavailability of several peptides and peptide-like drugs including most recently, epidermal growth factor (Rao et al., 1998) and a number of enkephalin (Krondahl et al., 1997; Langguth et al., 1997), oxytocin (Lundin et al., 1995) and thymopoietin analogues (Heizmann et al., 1996). Subsequent in vitro stability studies confirmed that GMDP was degraded by the luminal contents of the rat gastrointestinal tract (Fig. 4). GMDP was relatively stable in the stomach contents (and presumably, therefore, to pepsin-based degradation) but less stable in the contents of the small intestine, with ≈ 50% of the peptide degraded over the 4 h period tested. The caecum and large intestinal contents rapidly de-

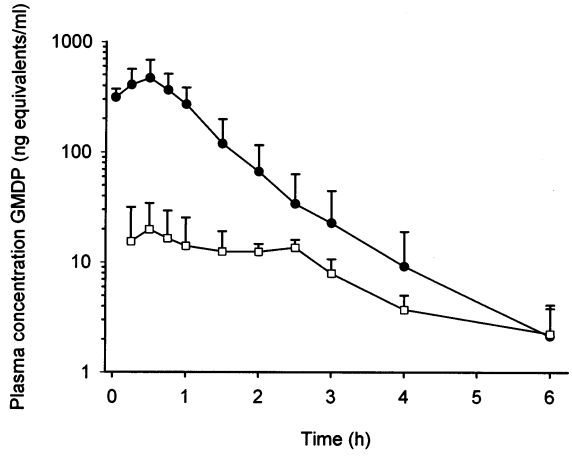

Fig. 5. The plasma concentration of GMDP following intraduodenal administration as a water-in-oil microemulsion formulation (●, mean ± S.D., $n = 4$) and by gavage as an aqueous solution (□, mean ± S.D., $n = 3$).

Table 2
Pharmacokinetic parameters for GNIDP after i.v., oral and intraduodenal administration of solution and microemulsion formulations (mean ± S.D.)

Dose (mg/kg)	Label	Route	Formulation	C_{max} (ng/ml)	$AUC^{0-\infty}$ (n·h/ml)	BA (%)
0.056	^{14}C	IV	Solution	N/A	82.2 ± 2.3[a]	N/A
0.45	^{14}C	Oral	Solution	22.8 ± 10.7	54.6 ± 28.9	8.2 ± 4.3
0.45	^{3}H	Intra-duodenal	Microemulsion	458.3 ± 147.6	531.6 ± 198.7	80.1 ± 30.4

[a] i.v. pharmacokinetics were not significantly different after administration of ^{3}H GMDP.

graded GMDP (Fig. 4). These in vitro data should also be viewed with the caveat that the ex-vivo GI contents used in the in vitro experiments were diluted with Ringer's solution in order to facilitate efficient removal from the GI tract and subsequent assay and therefore that the extent of luminal instability reported in the current studies is likely an underestimation of the true in vivo stability. In vitro permeability studies using isolated rabbit ileum were also conducted to determine the potential limitation to oral bioavailability posed by the passage of GMDP across the intestinal wall. Results indicate that GMDP is absorbed passively across the intestinal wall and does not appear to be a substrate of, for example, either the dipeptide active transport system (possibly due to the presence of the two sugar residues linked to the peptide backbone) or efflux pumps such as the *p*-glycoprotein efflux pump (Table 1). In common with many peptides (Burton et al., 1996; Nerurkar et al., 1996; Knipp et al., 1997) the absolute permeability of GMDP was low and lower than the permeability of the poorly absorbed hydrophilic marker mannitol.

The results of the bioavailability, in vitro luminal stability and intestinal permeability studies therefore, suggested that that oral bioavailability of GMDP was primarily limited by both degradation of the peptide while resident in the in the lumen of the gastrointestinal tract and low intestinal permeability.

In a subsequent attempt to improve the absorption of GMDP, water-in-oil microemulsion formulations were investigated as possible bioavailability enhancing vehicles. Water-in-oil microemulsions have previously been shown to significantly increase the bioavailability of hydrophilic peptides in rats (Constantinides et al., 1994; Constantinides, 1995; Constantinides et al., 1996) and it is thought that these systems function by protecting the compound from chemical/enzymatic breakdown in the intestinal lumen. The medium chain length fatty acid lipid components of the microemulsion system are also putative absorption enhancing agents and may therefore, increase the permeability of the intestinal wall to the peptide (Lundin et al., 1997; Yamamoto et al., 1997; Lindmark et al., 1998).

Delivery of GMDP in a water-in-oil microemulsion system increased the bioavailability of the peptide ten-fold from 8.3 ± 4.4% as an aqueous solution delivered by gavage to 80.1 ± 30.5% (mean ± S.D., $n = 3$). These data are consistent with previous studies which utilised a similar medium chain triglyceride based microemulsion to increase the oral bloavailability of the water soluble peptide SK&F 106760 and the marker compound, calcein, after intraduodenal administration to rats (Constantinides et al., 1994).

The results obtained when GMDP was delivered as a microemulsion were variable, but represent a significant increase in bioavailabilty when compared with the aqueous solution. The increase in bioavailability, however, must be interpreted carefully as the current study was conducted as a 'proof of concept' investigation and therefore under conditions specifically selected to maximise the likely impact of the microemulsion formulation. Thus, a relatively large volume of formulation (1 ml), was dosed directly into the duodenum of rats thereby limiting the chances of phase inversion and maximising the

protective and absorption enhancing properties of the formulation. Additional studies are required to determine if scaling the volumes and altering the delivery of the formulation to those more applicable to the human setting would negate the increase in bioavailability observed.

In conclusion the results of the current study indicate that the bioavailabilty of GMDP is limited by degradation of the peptide in the lumen of the gastrointestinal tract prior to absorption and low intestinal permeability. Protecting the molecule from degradation and enhancing the intestinal permeability of the compound via formulation in a water-in-oil microemulsion successfully increased the bioavailability of GMDP up to ten-fold compared with a simple solution. These data suggest that similar formulation approaches utilising w/o microemulsions may provide benefit in terms of oral bioavailability for molecules where luminal instability and low intestinal permeability limit bioavailability. Further work is required to determine whether dilution on oral administration may reduce the reported bioavailability enhancing effects.

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