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Research Paper

In Vivo Performance of an Oral MR Matrix Tablet Formulation in the Beagle Dog in the Fed and Fasted State: Assessment of Mechanical Weakness

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Purpose. To evaluate the behaviour of an oral matrix modified release formulation in the canine gastrointestinal tract, and establish if a mechanical weakness previously observed in clinical studies would have been identified in the dog model.
Materials and Methods. *In vitro* release profiles were obtained for two modified release matrix tablets containing UK-294,315, designed to release over either 6 (formulation A) or 18 (formulation B) hours. Tablets were labelled with ^{153}samarium and *in vivo* pharmacoscintigraphy studies were performed in four beagle dogs in the fasted state for both formulations, and following ingestion of an FDA high fat meal for formulation B.
Results. The matrix tablet formulations displayed significantly different *in vitro* release profiles (F_2<50), with time to 80% release for formulation A and B of 406 and 987 min respectively. Complete *in vivo* disintegration occurred at 339±181 and 229±171 for formulation A and B respectively in the fasted state, and at 207±154 min for formulation B in the fed state, in disagreement with *in vitro* release.
Conclusion. The fed/fasted dog model would have predicted a lack of physical robustness in the matrix tablet formulation B, however it would not have predicted the clear fed/fasted effects on performance observed previously in man.

KEY WORDS: food effects; gamma scintigraphy; IVIVC; matrix tablet; pharmacoscintigraphy.

INTRODUCTION

Oral controlled release delivery systems continue to remain an important tool in the current pharmaceutical market, allowing manipulation of a pharmaceutical compound to provide a formulation which may make it more convenient for a patient to take their medication, for example reducing the necessity for multiple administrations to once or twice daily dosing, or by providing more constant systemic exposure for a compound with a narrow therapeutic index. The performance of such controlled release dosage forms must be carefully studied in order to confirm consistent performance, on one hand ensuring that adequate drug release is achieved, and on the other hand avoiding the phenomenon of 'dose dumping,' where a larger than anticipated amount of drug is released as a bolus, resulting in dangerously high blood levels in the patient (1).

In the development of oral controlled release formulations *in vitro* characterisation is an extensive part of the optimisation process, with dissolution equipment testing remaining the main method employed by researchers to establish drug release profiles. Increasingly, it has been recognised that there are limitations to the standard *in vitro* release tests, as while they are an effective method for comparison and ranking of potential formulations, they are not always representative of conditions *in vivo*. Recently, researchers have reported attempts at simulating *in vivo* conditions *in vitro*, with methods ranging from adapting the standard dissolution apparatus for use with bio-relevant media (2,3), evaluating hydrodynamic flow (4), and constructing a synthetic gastrointestinal (GI) tract, incorporating bio-relevant media along with physical movements and residence times (5). Where clinical performance is known, is also possible to optimise release studies to match *in vivo* performance, avoiding the need for further bioequivalence studies during formulation optimisation through an IVIVc. While such methods may

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ABBREVIATIONS: ANOVA, Analysis of variance; AUC, Area under the curve; $AUC_{(0-8)}$, Area under the plasma concentration curve from 0–8 h.; $AUC_{(0-\infty)}$, Area under the plasma concentration curve from 0 h extrapolated to infinity.; CD, Complete tablet disintegration time; C_{max}, Maximum plasma concentration; GEC, Gastric emptying complete; GI, Gastrointestinal; HPMC, Hydroxypropylmethylcellulose; ID, Initial tablet disintegration time; IV, Intravenous; K_{el}, Elimination rate constant; MBq, Megabequerel; MMC, migrating myoelectric complex; MR, Modified release; ROI, Region of interest; SIT, Small intestinal transit time; Sm, Samarium; ^{152}Sm, samarium oxide-152; Tc, Technetium; T_{max}, Time to reach maximum plasma concentration; $T_{50\%}$, Time to 50% of parameter; $T_{1/2}$, Plasma half life; ^{99m}Tc-DTPA, Technetium-99m-diethylenetriaminepentaacetic acid.

represent an improvement for the investigation of oral controlled release formulations, as of yet there is no generally accepted *in vitro* model which can predict *in vivo* behaviour for any formulation, and it therefore becomes ultimately necessary to evaluate the dosage form *in vivo*, in order to establish actual performance.

The dog is currently a commonly used animal model in the development of such formulations (6–9), as it allows for administration of a dosage form with identical dimensions to that intended for clinical use, an important prerequisite in the case of modified release preparations. Differences between canine and human gastrointestinal tract parameters are well established and documented, and while gastric emptying rates may be relatively similar in the two species, one of the most striking differences with implications for extended release oral formulations is the short length of the small and large intestine, and reduced overall GI transit time in the dog compared to man (10,11). This may have implications for a compound which is absorbed predominantly from the upper GI tract, potentially leading to underestimation of bioavailability due to rapid transit time, or for a formulation that is intended for extended release or colon targeting in humans passing through the canine GI tract before drug release is complete. Some researchers have attempted to overcome this discrepancy with the use of agents which decrease gastrointestinal motility such as loperamide (12), although introducing an unnecessary drug compound into such studies is neither ideal for the animal, nor for the validity of any pharmacokinetic data. The pH of the canine stomach, both basal and in response to consumption of food is documented as being either similar to or higher than that of man, although there may be more variability in the dog (10,13), and the pH of the canine small intestine is generally reported as being approximately one pH unit higher at any equivalent point of the human GI tract (14). Other areas where differences can be observed between the two species which may affect pharmaceutical formulations are in the enzyme content of the gut, difference in apparent pylorus aperture potentially prolonging gastric retention of monolithic dosage forms in the dog, higher destructive mechanical forces in the dog, and the suggestion that the intestinal permeability is higher in dogs (15,16), leading to the potential for misleading drug absorption data. It is also suggested that while the canine GI tract has a similar migrating myoelectric complex (MMC) periodicity and cycle length to man, the canine stomach behaves differently in response to food, with an extended gastric emptying time, and a longer lag time before recovery of the MMC cycle and phase III 'housekeeper' wave (10).

The literature therefore reports caution when extrapolating results obtained in the dog to predicted results in man (2,17), with examples reported of both over- and underestimation of drug absorption in the canine model. A report of a retrospective study of 43 different non-dissolution rate limited compounds in the dog suggested a very low correlation between bioavailability in dog and man, which was suggested to be a result of the many physiological differences (16). However, in the absence of adequate *in vitro* tests, the dog often remains the initial *in vivo* investigational tool for a novel oral controlled release dose, and other researchers have reported the dog as a useful animal model (18), some suggesting gastric emptying in the dog is closer to man than for other animal models (19,20). However, much of the data gathered to date has been inferred from pharmacokinetic data, rather than direct observation of dosage location using a technique such as gamma scintigraphy.

Gamma scintigraphy has long been established as the 'gold standard' as a non-invasive means of imaging the *in vivo* behaviour of pharmaceutical dosage forms in man, its use widely reported in the literature (21–23), and has been used to study *in vivo* erosion rates of Hydroxypropylmethylcellulose HPMC tablets (24). If applied to the canine model, gamma scintigraphy may represent a means of improving understanding and interpretation of results obtained in terms of their relevance to man (25). The use of gamma scintigraphy in the dog has been previously reported as a technique for monitoring transit and oral dosage form performance in this animal model by only a few researchers (26–28), and may provide valuable information on the location of a dosage form at the time of a particular pharmacokinetic event in the plasma profiles. This would allow more detailed interpretation of the pharmacokinetic profiles, allowing a particular event, e.g. change in absorption rate, to be explained by the location or behaviour of the dose at that particular time.

There are various reports in the literature of the effects of food on oral formulations (29–31). A previous clinical pharmacoscintigraphic study of a matrix controlled release formulation of an investigational compound UK-294,315, which had displayed robust 18 h controlled release *in vitro* for a 100 mg dose, established a dose dumping effect when co-administered with a high fat meal, but not in the fasted state (unpublished data). The purpose of the current investigation was to investigate this formulation in the commonly used beagle dog model, under similar conditions to the clinical study, to establish if a mechanical weakness in the formulation would have been identified, and if any correlation of the behaviour of this formulation could be observed between the two species. The 18 h controlled release formulation was therefore studied in the fasted and fed state in the dog, using exactly the same test meal as was given to the human volunteers. A 6 h controlled release formulation (20 mg dose) which had been previously shown to be robust in human volunteers in both fed and fasted conditions (unpublished data) was also studied in the fasted state, in order to establish if a formulation of this compound known to perform well in man could also perform well in the dog.

MATERIALS AND METHODS

Materials

UK-294,315[1] (phosphate salt form was used—UK-294,315-09, pKa 8.5, LogP 1.8, aqueous solubility in water 0.74 mg/ml, F=0.71 in the dog) and UK-298,108[2] were obtained from Pfizer Global Research and Development (Sandwich, UK), Hydroxypropylmethylcellulose (Methocel) was purchased from Dow Chemicals (Michigan, USA), lactose monohydrate (Fast-flo) was from Foremost (New Jersey, USA), Aerosil 200 was from Degussa (Dusseldorf, Germany), magnesium stearate from

[1] 4-Amino-5-(4-fluorophenyl)-6,7-dimethoxy-2-[4-(morpholinocarbonyl)-perhydro-1,4-diazepin-1-yl]quinoline

[2] 4-amino-5-(4-chlorophenyl)-6,7-dimethoxy-2-[4-(morpholinocarbonyl)-perhydro-1,4-diazepin-1-yl]quinoline

Peter Greven (Venlo, Netherlands), and povidone (Kollidon) from BASF (Cheshire, UK). Isotopically enriched samarium oxide-152 (^{152}Sm) was purchased from CK Gas (Hampshire, UK). Lithium heparin beaded monovette blood sample tubes and luer adaptors were obtained from Sarstedt (Leicester, UK). Steriflex 1, 0.9% w/v sodium chloride Intravenous Infusion BP was purchased from Fresenius

Kabi Ltd. (Warrington, UK), Sterifix filters with a pore size of 0.2 μm were obtained from B. Braun (Sheffield, UK). Potassium dihydrogen orthophosphate (KH_2PO_4), ammonia, acetonitrile, ethyl acetate, methanol and acetic acid were purchased from VWR (Lutterworth, UK), and 1-octane sulphonic acid was purchased from Sigma Aldrich (Poole, UK). Technetium-99m-diethylenetriaminepentaacetic acid (^{99m}Tc-DTPA) was obtained from the Radionuclide Dispensary (Glasgow, UK), and Isolute HCX SPE (10 ml/130 mg) cartridges were obtained from Esslab (Essex, UK).

Methods

Manufacture of UK-294,315 Containing Tablets

Twenty milligrams modified release (MR) tablets (formulation A) designed to release drug over 6 h contained UK-294,315 (20 mg), HPMC, lactose, Aerosil, magnesium stearate, and samarium oxide (6 mg), and were manufactured using conventional dry blending processes followed by compression to circular, convex tablets (8 mm diameter, approximately 9 Kp hardness). One hundred milligrams MR tablets (formulation B) designed to release drug over 18 h contained UK-294,315 (100 mg), HPMC, lactose, povidone, and magnesium stearate, and were manufactured using conventional wet granulation processes, prior to samarium oxide (6 mg) extragranular addition and compression to circular, convex tablets (9.5 mm diameter, approximately 10.5 Kp hardness). ^{152}Sm is a stable isotope which upon neutron activation can be transformed into the radioisotope ^{153}Sm, a gamma emitter with a half life of 46.3 h. Enriched samarium oxide was used in order to avoid the low radioactive yields associated with natural abundance samarium (40). When required for study, tablets containing ^{152}Sm were irradiated for 2.5 min in a neutron flux of 0.8 E^{12} n/cm^2/s, to allow approximately 1 MBq of activity at time of dosing.

Manufacture of UK-294,315 Intravenous (IV) Dose

Solutions of 0.5 mg/ml UK-294,315 were prepared in 0.9% w/v sodium chloride Intravenous Infusion BP, to give a dose of 0.5 mg/kg for each animal.

In Vitro Release

Release studies were performed using a USP XXIII basket dissolution apparatus (Caleva, Dorset, UK) at 100 rpm, with 1,000 ml pH 7.5 media (0.06 M KCl/0.03 M NaCl/0.006 M KH_2PO_4/0.005 M NaOH) at a temperature of 37°C. The main purpose of the *in vitro* release study was to compare irradiated and non irradiated tablets to gain confidence that irradiation would not affect release, and also to ensure that tablets manufactured for the current study performed in a similar manner to the previous work. Therefore the dissolution conditions used were identical to those previously used in the clinical study to allow direct comparison of data. Samples were taken at 1 h intervals for a period of 24 h using an automated sampling system, and absorbance in cells of path length 2 mm was measured using a Cecil CE 3021 UV spectrophotometer (Cecil Instruments, Cambridge, UK).

In Vivo Study

All procedures were performed under a valid UK Home Office Animals (Scientific Procedures) Project Licence, and according to the Principles of Laboratory Animal Care. Four male beagle dogs (11–14 kg) were dosed on separate occasions, with a washout period of at least 1 week between studies. The animals were fasted overnight prior to each study, with free access to water. At the start of each study day involving active drug administration a temporary cannula was inserted into the saphenous vein in the hindleg to facilitate blood sampling during the study period. The cannula was kept patent by flushing with heparinised normal saline when required, and was removed at the end of the study day, with any further blood samples required being collected by venepuncture.

Two preliminary study legs were carried out where the animals were given either 2 ml/kg water labelled with 5 MBq ^{99m}Tc, or an FDA high fat meal labelled with 2 MBq ^{99m}Tc, in order to evaluate basic transit rate and gastric emptying parameters of the dogs. On further separate occasions, formulation A was dosed orally in the fasted state, and formulation B was given orally in the fasted state and the fed state. For fasted studies the animals were allowed access to water *ad libitum* during the study period, and food was allowed when the formulation was seen to have entered the colon (generally around 4 h post dose, consistent with the protocol used for the clinical study). For fed studies, the animals were fed a meal equivalent to the human FDA high fat meal for bioequivalence studies approximately 10 min before the time of dosing. The meal consisted of two strips bacon, two slices toast (+15 gm butter), 100 gm hash browns, 100 gm scrambled eggs (two eggs, 20 ml whole milk, 15 gm butter) and 200 ml whole milk, and provided approximately 1,000 calories, 50% of which were from fat. The calorific and fat content of this meal was identical to that used in the previous clinical investigation, and in each case the meal was eaten by the dog in less than 5 min. A standard canine meal was given approximately 8 h following dosing. An intravenous (IV) dose was given in the fasted state on a separate occasion, with food allowed 4 h post-dose.

On each study occasion the activity of the tablet was confirmed by measuring in a dose calibrator, before administration to the animal. Markers containing approximately 0.1 MBq ^{99m}Tc were placed at the centre of the shoulder blades, and at the base of the tail for positional reference between images. For dosing, the animal was placed in a sling which allowed the dog to remain in an unrestrained standing position, while providing support to allow alignment of the animal. Tablets were administered using a pill dosing device, and immediately following oral dosing the animal was placed under the gamma camera for imaging. The animals had been previously trained to stand comfortably in the slings, and when

the imaging interval became less frequent (e.g. after gastric emptying) the animal was allowed to roam the floor or placed in a holding pen, allowing a similar degree of mobility as would be expected from a human volunteer, thereby minimising any undue effect on GI transit through immobility. The IV dose, which was not radiolabelled, was administered through a 0.2 μm filter over a period of approximately 1 min, via a cannula placed in the saphenous vein of the hindleg which was not being used to obtain blood samples.

Blood samples of 5.5 ml were collected into pre-labelled heparin beaded tubes at time points 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8 and 10 h for oral doses, and at 0, 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2 3, 4, 6, 8, 24 h for the IV dose. Due to experimental constraints (access to facilities) it was not possible to obtain 24 h samples for the oral doses, however, this study was not designed to elucidate pharmacokinetic behaviour of the compound but the performance of the actual formulation. Blood samples were centrifuged at 2,000 rpm at 4°C for 15 min, within 60 min of sample collection time. Plasma was then separated from the sample, and transferred to pre-labelled containers within 60 min of centrifugation for storage at −20°C, until required for analysis.

Imaging using a gamma scintigraphic camera with a field of view of 533×387 mm, equipped with a low energy parallel hole collimator (MIE Systems, Germany) was carried out from the posterior aspect every 2 min post dose until the tablet was seen to empty from the stomach or disintegration was complete, every 15 min until the activity was seen to enter the colon, and then every 30 min until the end of the study. Images were stored electronically for subsequent analysis.

Scintigraphic Data Analysis

Scintigraphic images were analysed with Scintron analysis software (MIE Systems, Germany). Using the positional markers as reference and comparing all individual images, regions of interest (ROI) were drawn around the tablet, stomach, caecum, and colon to assess the counts in that area and the data was corrected for background activity and radioactive decay. For the labelled liquid and high fat meal, parameters evaluated were onset and completion of gastric emptying. Parameters evaluated for the tablets were time of gastric emptying, small intestinal transit time, colon arrival, initial disintegration, and complete disintegration. Gastric emptying was defined as the time midway between the image where the tablet core was last seen in the stomach, and the image where the tablet core was first observed in the small intestine, with similar parameters applied to colon arrival time. Where the tablet completely disintegrated in the stomach, gastric emptying was recorded as the time of onset and complete emptying of the disintegrated tablet. Small intestinal transit (SIT) was defined as the time period between tablet core gastric emptying time and colon arrival for the tablet formulations. Mean total time for tablet disintegration was determined by subtracting the initial disintegration from the complete disintegration time for each animal.

Initial tablet disintegration was defined as the time point midway between the last image where no tablet disintegration was observed, and the first image where evidence of disintegration of the tablet core was observed in two or more consecutive images. Complete tablet disintegration was determined as the time point at which the presence of a distinct tablet core could no longer be detected. Scintigraphic data was tested for significance using analysis of variance (ANOVA).

Plasma Analysis

Plasma samples were defrosted at room temperature and prepared by adding 30 μl of internal standard (UK-298,108) at a concentration of 10 μg/ml to 1 ml of plasma, followed by 2 ml 0.1 M KH_2PO_4 (pH 6), and vortex mixing briefly. Calibration standards were prepared by using blank dog plasma and adding known amounts of UK-294,315. Isolute HCX SPE cartridges were activated with 2 ml methanol, followed by 2 ml 0.1 M KH_2PO_4 (pH 6). The prepared plasma sample was then passed slowly through the cartridge. The cartridge was then washed with 1 ml acetic acid (1 M), and dried under a full vacuum for 5 min, then washed with 2 ml methanol, and dried under full vacuum for a further 2 min. Finally the sample was eluted with 2 ml 2% ammonia in ethyl acetate (made up freshly on each day of use) into a clean glass vial. The sample was evaporated to dryness and re-dissolved in 120 μl of HPLC mobile phase. HPLC mobile phase consisted of 40% acetonitrile: 60% (50 mM KH_2PO_4 + 10 mM 1-octane sulphonic acid, pH 3), with a flow rate of 1 ml/min and UV detection at 255 nm. The HPLC assay was carried out using a Thermo Separation Products HPLC system with Spectra System UV 1000 detector, and a HiChrom RPB (5 μm, 25 cm× 4.6 mm) column. Resultant chromatograms were analysed using ChromQuest software (Thermo Electron Corporation). The limit of detection was 1 ng/ml.

Pharmacokinetic Parameters

Pharmacokinetic parameters for each animal were evaluated using WinNonlin v 5.0.1 analysis software (Pharsight), with a non-compartmental model for the oral data. Parameters evaluated for the oral MR formulations were C_{max}, T_{max}, area under the curve (AUC), and for the IV formulation AUC, K_{el} and $T_{1/2}$. Bioavailability of the oral doses relative to IV administration was calculated (F_{rel}) using the AUC from 0–8 h (AUC_{0-8}), as in many instances the elimination phase had not occurred by the end of the sampling period for the oral MR formulations. The purpose of estimating bioavailability from data up to 8 h is to compare the performance of the MR products with observed scintigraphic behaviour data over the timeframe of the experimental procedure, despite the fact that the values reported will not be absolute as the elimination phase may not have started.

The AUC extrapolated to infinity ($AUC_{-\infty}$) was also calculated for the IV dose. Basic pharmacokinetic parameters were compared using one-way ANOVA, and deconvolution analysis based on a two compartment IV model (assessed from *in vivo* data) was performed on the oral plasma data, using WinNonlin software.

RESULTS

Release Studies

The release profiles for the two oral formulations are shown in Fig. 1, and demonstrate that there was a significant

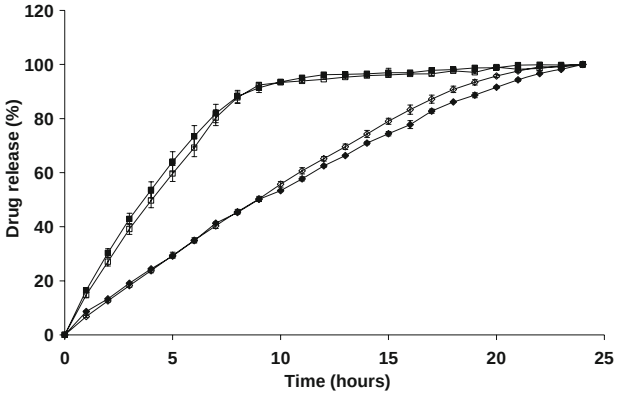

Fig. 1. Profiles showing release of UK-294,315 (at pH 7.5, 37°C, 100 rpm) from formulation A (*square*) and B (*diamond*), before (*solid symbols*) and after (*open symbols*) activation by neutron irradiation.

difference in the two formulations, with formulation A releasing drug more rapidly than formulation B (similarity value F_2=33, reference formulation A). The time to 80% total drug release was approximately 7 h for formulation A, and approximately 17 h for formulation B. Previous studies have reported the deleterious effects of the neutron activation process on the performance of oral formulations (32,33), in particular the breakdown of the matrix forming polymer component. Therefore in this study isotopically enriched samarium oxide was used in order to minimise the required neutron activation time to obtain the desirable level of radioactivity. The release profiles of the two formulations following neutron irradiation demonstrated that the activation process did not affect the release behaviour of either formulation, and that the profiles obtained were not significantly different from the profiles prior to irradiation (similarity values (F_2) of 69 and 85 for formulations A and B respectively, with tablets before irradiation as reference in each case).

Scintigraphic Analysis

Table I shows the GI transit and tablet disintegration data assessed using gamma scintigraphy. The radiolabelled liquid emptied rapidly from the stomach (Fig. 2), and in each case gastric emptying had already begun by the time the first image was acquired. It was observed that while the majority of the liquid had cleared from the stomach within 15 min, in some dogs a residual amount remained for a longer period of time, giving a mean time to complete gastric emptying for the liquid of 86.4±80.6.

The mean time to observed onset of gastric emptying of the high fat meal was 67.1±25 min, with evidence of some activity still remaining in the stomach ROI (<10% of initial counts) at the completion of the study day (480 min). Results previously reported in the literature for gastric emptying time of a standard dog meal were 285 min (26), although in this case the fat and energy content of the meal was lower at 6% and 450 calories respectively. The mean gastric emptying profiles are shown in Fig. 2, and show that up to 2 h there is very little variability in the gastric emptying data, while after this point the variability increases.

The average time to initial disintegration of formulation A (20 mg) in the fasted state was 45.4±14 min, with tablet disintegration complete at 339±181 min, which is in a similar range to the release profile for this formulation which showed 80% release at approximately 406 min. The *in vivo* behaviour of formulation B however did not reflect the *in vitro* release profile. In the fasted state, initial tablet disintegration of formulation B (100 mg) was observed to begin at 47.4±40 min, similar to formulation A, however the average time to complete disintegration *in vivo* was 229±171 min, shorter than for formulation A, reversing the trend observed in the release profiles where 80% release was observed at 987 min. In the fed state for formulation B (100 mg), the time to initial tablet disintegration was 56.4±34.8 min, slightly slower than for the fasted state. The time to complete tablet disintegration was 207±154 min, slightly faster than for the fasted state. The average total time for tablet disintegration for formulation A (20 mg) was much longer at 294 min than for formulation B in either the fasted or fed state (181 and 150 min respectively), which is in complete disagreement with the profiles obtained from the *in vitro* release study.

For formulation A (20 mg), there were two instances where initial tablet disintegration was in the stomach, and

Table I. Scintigraphic Gastrointestinal Transit Data in Dogs (Mean ± s.d., *n*=4, Unless Otherwise Specified)

Orally Administered	Tablet Disintegration		Gastric Emptying			Transit	
	Initial (min)	Complete (min)	Tablet Core (min)	Onset (min)	Complete (min)	Colon Arrival (min)	SIT (min)
Water	N/A	N/A	N/A	< 1	86.4±80.6	N/A	N/A
High fat meal	N/A	N/A	N/A	67.1±25	> 480	N/A	N/A
Formulation A	45.4±14	339±181	36.8±57[a] (*n*=3)	73[b] (*n*=1)	232.5[b] (*n*=1)	106.2±12.8[c] (*n*=2)	102.3±13.8[c] (*n*=2)
Formulation B (fasted)	47.4±40	229±171	23[a] (*n*=1)	52.7± 11[b] (*n*=3)	178±86.5[b] (*n*=3)	206.5[c] (*n*=1)	183.5[c] (*n*=1)
Formulation B (fed)	56.4±34.8	207±154	N/A	81.5±32[b]	267±181[b]	N/A	N/A

N/A Not applicable
[a] Gastric emptying of intact tablet core.
[b] Gastric emptying of disintegrated tablet.
[c] Transit of intact tablets only.

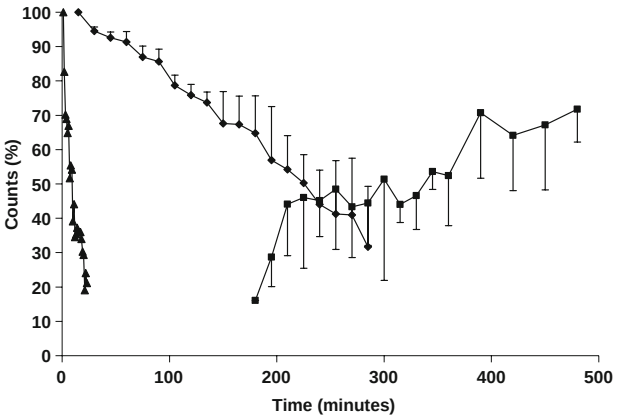

Fig. 2. Mean (± s.d.) scintigraphic profile (n=4) showing emptying of liquid (*triangle*) and food (*diamond*) from the stomach on separate occasions, followed by entry of food into the colon (*square*) (small intestinal counts omitted for clarity).

two instances where gastric emptying occurred rapidly, one before imaging had even begun (3 min), and one during image acquisition (5 min), indicating that the MMC cycle was probably in Phase III at this time. Initial disintegration of one tablet of the two rapidly emptied tablets was in the small intestine, and one in the caecum. Of the two tablets that began disintegrating in the stomach, one tablet completely disintegrated here, and one emptied from the stomach and completed disintegration in the small intestine. The two tablets which were rapidly emptied from the stomach completed disintegration in the colon.

In the fasted state formulation B began disintegration in the stomach in three instances, and in the small intestine in one (following relatively rapid gastric emptying at 23 min). Complete disintegration occurred in the stomach for two tablets, in the small intestine for a tablet which emptied from the stomach following initial disintegration and in the colon for the tablet that began disintegration in the small intestine. Therefore in the case of these particular formulations, tablets of diameter 8 mm (formulation A) and 9.5 mm (formulation B) were able to pass the pyloric sphincter of the dog intact.

In the fed state for formulation B (100 mg), all four tablets began and completed disintegration in the stomach, and the images suggested that the disintegrated tablet emptied gradually from the stomach with food, as the time to complete gastric emptying of the disintegrated tablet was longer in the fed state. Due to the rapid disintegration of formulation B in the stomach in the fed state, SIT of the tablet could not be calculated, and this parameter could only be calculated for one animal in the fasted state for this formulation, as there was only one instance of transit of an intact tablet core from stomach to colon. SIT could only be calculated for two animals for formulation A for the same reasons (Table I). Representative scintigraphic images are shown in Fig. 3.

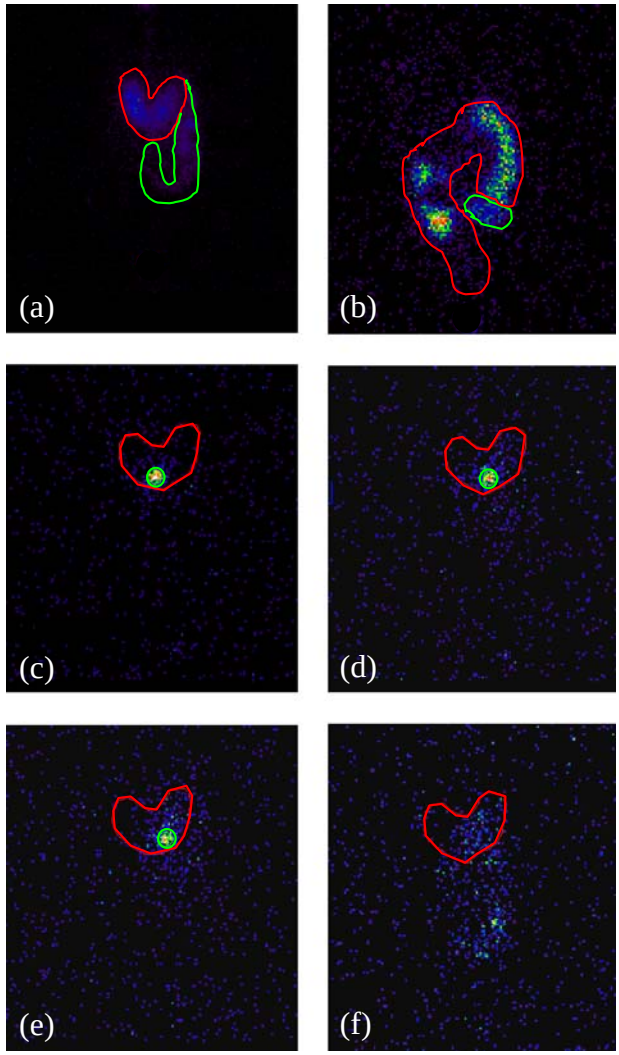

Fig. 3. Representative scintigraphic images showing **a** radiolabelled liquid in the stomach (*red*) and emptying into the duodenum (*green*), **b** radiolabelled liquid in the caecum (*green*) and colon (*red*), **c** intact tablet in the stomach, **d** initial tablet disintegration, **e** further tablet disintegration, and **f** complete tablet disintegration.

Plasma Analysis

The mean *in vivo* plasma profiles for UK-294,315 tablets are displayed in Fig. 4. All data are normalised to a dose of 2 mg/kg. Pharmacokinetic parameters are shown in Table II. Using the deconvolution data, a plot of cumulative drug

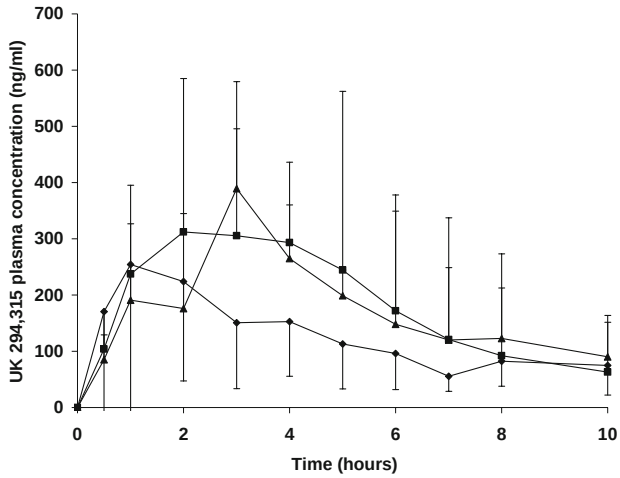

Fig. 4. Mean (± s.d.) plasma profiles of UK-294,315 normalised to a dose of 2 mg/kg following administration of formulation A (*diamond*), and formulation B in the fasted (*square*) and fed (*triangle*) state.

Table II. Pharmacokinetic Data for Oral Matrix Tablet Formulations, Normalised to a Dose of 2 mg/kg (Mean ± s.d.)

Formulation	Median T_{max} (range) (h)	C_{max} (ng/ml)	AUC_{0-8} (ng h/ml)	$AUC_{0-\infty}$ (ng h/ml)	K_{el} (h^{-1})	$T_{1/2}$ (h)	F_{rel}[a] (%)
IV	–	3,286±860	3,359±1279	3,552±1182	0.295±0.095	2.59±0.99	100
A	2.5 (3)	331±308	1,244±982	–	–	–	42.21±±28.9
B (fasted)	1.75 (2.5)	449±208	1,754±863	–	–	–	46.81±26.1
B (fed)	2.25 (2.5)	408±289	1,478±704	–	–	–	50.76±13.39

[a] Calculated using data for AUC_{0-8}

absorption was compared against the cumulative drug release from *in vitro* release tests, to establish any *in vitro in vivo* correlations. Comparing the *in vivo* input amount obtained by deconvolution of the plasma data with the *in vitro* release profiles (Fig. 5) shows that for formulation A *in vivo* release was slightly faster than or equal to the *in vitro* release rate for the first 2 h, following which the *in vivo* release rate was slower than *in vitro*. *In vivo* release of formulation B was faster than the *in vitro* release rate, in both the fed and fasted states.

DISCUSSION

The *in vivo* behaviour of two oral controlled release matrix tablets previously evaluated clinically was evaluated in the beagle dog, using pharmacoscintigraphic methods, and results were compared to the clinical investigation.

When assessing the oral matrix tablet formulations for disintegration rate, one drawback with the currently used method is that imaging was only undertaken from the posterior aspect in the dog. Commonly accepted procedure for assessing disintegration rate in a clinical study would require the acquisition of images from both anterior and posterior aspects, allowing correction for movement of the formulation, either towards or away from the camera. The restraints of the current study procedure, in particular the use of a standing sling design, meant that this was not practically possible. Therefore, while visually determined values such as initial and complete tablet disintegration time can be determined with reasonable accuracy, an overall disintegration profile will contain a degree of error due to the planar movement of the tablet as it travels down the GI tract. However, the evaluation of this parameter will still provide general useful information towards the behaviour of the formulation, bearing in mind the overall inaccuracy of the values.

Scintigraphic Data

The location in the GI tract of initial and complete disintegration of the matrix tablets provided insight into the overall *in vivo* behaviour of the two formulations. The general trend was that the disintegration of formulation A followed a controlled release pattern throughout the whole of the GI tract, whereas for formulation B, disintegration tended to occur towards the upper GI tract.

In the fed state, without exception formulation B disintegrated completely in the stomach, which could be construed to be as a result of the delayed gastric emptying that would be induced by the high fat meal. In the fasted state, the overall tendency was also for formulation B to disintegrate completely in the stomach or duodenum. This finding was surprising as formulation B was observed to release over 17 h *in vitro*, and had previously shown robust extended release performance in fasted volunteers clinically (unpublished data). It may be possible that the larger diameter of this tablet (formulation A=8 mm, formulation B=9.5 mm) made gastric emptying of an intact tablet less likely to occur, however subsequent studies by our group (data not shown) have demonstrated gastric emptying within 26–47 min for tablets of 13 mm diameter in the dog.

In the one instance of rapid gastric emptying of formulation B in a fasted dog (23 min), disintegration began in the small intestine and completed in the descending colon. The

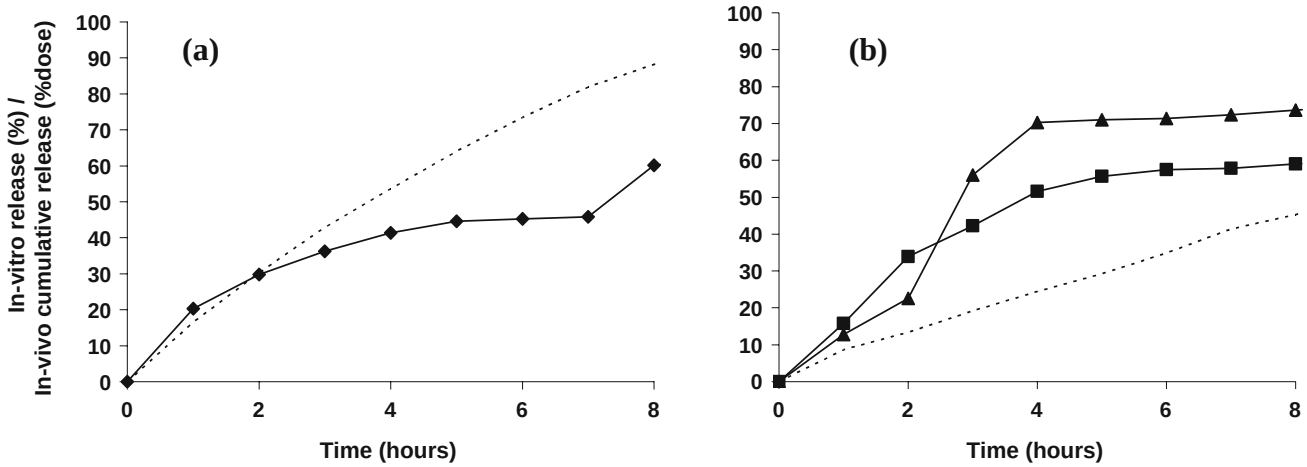

Fig. 5. Comparison of *in-vitro* release with *in-vivo* absorption. Release profiles represented by dashed line. **a** formulation A (*diamond*), **b** formulation B in the fasted (*square*) and fed states (*triangle*).

time to complete disintegration in this one instance was 450 min, compared to the mean value of 229 min, suggesting that the stomach is the region of the canine GI tract that is placing the highest level of strain on the formulation. This theory may also be corroborated by the disintegration data for formulation A, where in two instances the tablets were observed to empty extremely rapidly from the stomach (at 3 and 5 min), and in both cases the total tablet disintegration time was longer than for the two tablets which remained in the stomach for a greater period of time.

The canine stomach therefore appears to be an equally harsh environment for either formulation in both the fed and fasted states. These findings are similar to that reported by Kamba *et al.* (25,34), who found that in the dog the forces acting on an oral formulation in the stomach were much higher than in the small intestine, and that the forces in the stomach were unaltered by the presence of food (25).

The time to complete disintegration was longer for formulation A than formulation B (although not statistically significant), suggesting that following the initial release formulation A disintegrates more slowly than formulation B. This is a reversal of the disintegration behaviour which was predicted by the *in vitro* release profiles.

From the scintigraphic data for formulation B, it was observed that the average initial and complete gastric emptying times of the disintegrated tablet were delayed in the fed state, although no statistically significant difference was detected.

The average onset of emptying of the disintegrated portion of formulation B was at 81.5±32 min, agreeing well with the average time of initial gastric emptying of the high fat meal at 67.1±25 min. The average time to complete gastric emptying of the disintegrated tablet (formulation B) in the fed state was shorter than for the labelled high fat meal alone, and it is assumed that the small particles of samarium in the tablet were emptied ahead of the food which was still being ground down, as suggested in the literature (35,36).

SIT became difficult to accurately quantify in this study, because of the number of occasions where complete tablet disintegration had occurred before the tablet had reached the caecum/colon.

Pharmacokinetic Data

The UK-294,315 plasma levels were initially evaluated in conjunction with the scintigraphic data for each animal and formulation individually, following which the mean plasma profiles were considered (Fig. 4). Figure 6 shows plasma profiles of two animals for the two oral formulations, and points of pharmacokinetic interest defined and explained by the scintigraphic images.

The mean plasma T_{max} values (Table II) for the oral formulations A and B were similar ($P>0.05$), irrespective of the fasted or fed state. This may partially reflect the fact that the scintigraphic data showed a similar time to initial disintegration for the oral formulations. A previous *in vivo* study of this compound in the dog (37) reported complete absorption (71% bioavailability) and linear pharmacokinetics up to 10 mg/kg, suggesting that the main determinant of absorption in the current study would be the availability of the compound from the modified release dosage form.

C_{max} values for the oral doses were not significantly different from each other. Visually however, the data suggests that at 331 ng/ml the average C_{max} for formulation A was slightly lower than for formulation B in both the fasted and fed states (449 and 408 ng/ml respectively), which suggests that the dose dumping of formulation B observed in the fasted and fed state produced a higher peak in blood plasma

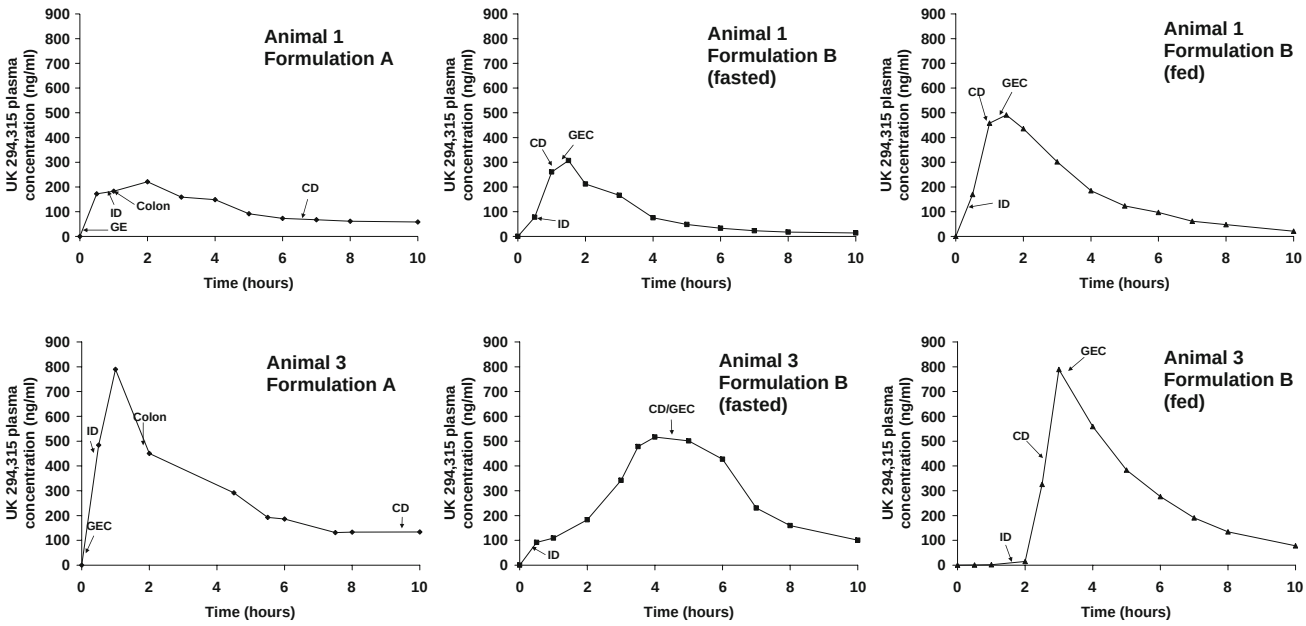

Fig. 6. UK-294,315 plasma profiles normalised to a dose of 2 mg/kg for two animals (1 and 3) with scintigraphic events following administration of formulation A (*diamond*), and formulation B in the fasted (*square*) and fed (*triangle*) state: Initial tablet disintegration (*ID*), complete tablet disintegration (*CD*), gastric emptying complete (*GEC*), tablet colon arrival (*Colon*).

levels. The suggestion that the more rapid disintegration rate of formulation B may have produced an effect on the plasma profiles is further supported by the AUC data, which while not statistically significant, shows higher average AUC values than for formulation A.

General Discussion

While formulation A displayed controlled release characteristics in the dog, as it had previously in man, the results obtained in the dog for the disintegration of formulation B in the fasted state did not correlate well with observations in man (unpublished data). A driving factor for the current investigation was that while this formulation appeared to disintegrate in a controlled release manner in fasted human subjects, the consumption of a high fat meal drastically accelerated the tablet erosion and disintegration rate. It was concluded that the difference in integrity observed was due to increased gastric residence time and exposure to hydrodynamic effects and higher mechanical destructive forces in the fed human stomach, although it was observed that despite this there was no corresponding increase in total amount of drug absorbed (AUC). The observation in the current study however, was that while formulation B appeared to dose dump in the fed state, dose dumping also occurred in the fasted state, suggesting that there may be a difference between the behaviour of the fasted stomach in dog and man.

The difference between dog and man in the forces acting on the fasted stomach has been previously implied by the data reported by Kamba *et al.* (38), who showed that the forces measured in the stomach of fasted human subjects was 1.5 N, while in the dog the force was 3.2 N irrespective of the fed or fasted state. The force of the fed stomach in man was measured at 1.9 N, less than for the canine stomach, which suggests that in the case of formulation B, there may have been a very fine tolerance level of stress, which lay somewhere between the compression forces of the human fasted and fed stomach, but was easily overcome by the increased forces in the canine stomach. Therefore in the case of this particular formulation, the canine stomach may have predicted the dose dumping effect of the controlled release formulation in man in the fed state, however it would not have been predictive of the fed/fasted effect seen in man.

Despite the discrepancies observed between the fed and fasted states in dog and man, the conclusion reached following the earlier clinical study that the results from the clinical pharmacoscintigraphic investigation of formulation B "clearly identified the need for further optimisation" to improve robustness under the stressful conditions of the fed stomach (unpublished data), would also have been the general conclusion of the current study in the dog, as the results undoubtedly identified the poor performance of formulation B. It is important to remember that such findings may only be valid for the particular formulation studied, for example previous reports of controlled release tablets susceptible to GI destructive forces in man found that food did not have an effect on tablet disintegration (1), and in both fasted dog and man formulation A was observed to be robust. However, based on the current findings, the dog model can be considered valuable in testing the mechanical robustness of modified release formulations. Other researchers also previously concluded that the dog model may be useful for evaluating the performance of MR products in man, providing a better prediction of the range of difference in two formulations in humans, than was obtained from *in vitro* evaluation (39).

It is beyond the scope of this investigation to speculate on the reason for the failure in performance (dose dumping) of formulation B, however *in vitro* studies are currently underway to investigate the mechanism of mechanical weakness.

CONCLUSION

A matrix tablet formulation designed with an 18 h controlled release profile (Formulation B) disintegrated more rapidly than a 6 h release formulation (Formulation A), in both the fasted and fed canine stomach, evaluated by considering plasma data in conjunction with scintigraphic images. The findings for formulation B in the fasted dog were in contrast with a previous clinical scintigraphic study where the formulation appeared to behave as desired in the fasted state, but dose dumped in the fed human stomach. In the case of this particular formulation it therefore appears that the canine stomach may have been a more severe test than that of the fasted human stomach, and that the fasted dog model would have predicted the failure of the formulation in the fed human stomach and the need to improve robustness, but would not have predicted the fed/fasted effect seen in man.

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REFERENCES

1. M. Shameem, N. Katori, N. Aoyagi, and S. Kojima. Oral solid controlled release dosage forms: role of GI-mechanical destructive forces and colonic release in drug absorption under fasted and fed conditions in humans. *Pharm. Res.* **12**(7):1049–1054 (1995).
2. L. Kalantzi, B. Polentarutti, T. Albery, D. Laitmer, B. Abrahamsson, J. Dressman, and C. Reppas. The delayed dissolution of paracetamol products in the canine fed stomach can be predicted *in-vitro* but it does not affect the onset of plasma levels. *Int. J. Pharm.* **296**:87–93 (2005).
3. T. Goto, N. Tanida, T. Yoshinaga, S. Sato, D. J. Ball, I. R. Wilding, E. Kobayashi, and A. Fujimara. Pharmaceutical design of a novel colon-targeted delivery system using two-layer-coated tablets of three different pharmaceutical formulations, supported by clinical evidence in humans. *J. Control. Release* **97**:31–42 (2004).
4. N. Katori, N. Aoyagi, and Terao. Estimation of agitation intensity in the GI tract in humans and dogs based on *in-vitro/in-vivo* correlation. *Pharm. Res.* **12**(2):237–243 (1995).
5. S. Blanquet, E. Zeijdner, E. Beyssac, J. Meunier, S. Denis, R. Havenaar, and M. Alric. A dynamic artificial gastrointestinal system for studying the behaviour of orally administered drug dosage forms under various physiological conditions. *Pharm. Res.* **21**(4):585–591 (2004).
6. N. Venkatesan, J. Yoshimitsu, Y. Ohashi, Y. Ito, N. Sugioka, N. Shibata, and K. Takada. Pharmacokinetic and pharmacodynamic studies following oral administration of erythropoietin mucoadhesive tablets to beagle dogs. *Int. J. Pharm.* **310**: 46–52 (2006).
7. T. Ishibashi, H. Hatano, M. Kobayashi, M. Mizobe, and H. Yashino. *In-vivo* drug release behaviour in dogs from a new

colon-targeted delivery system. *J. Control. Release* **57**:45–53 (1999).
8. Y. Wu, A. Loper, E. Landis, L. Hettrick, L. Novak, K. Lynn, C. Chen, K. Thompson, R. Higgins, U. Batra, S. Shelukar, G. Kwei, and D. Storey. The role of biopharmaceutics in the development of a clinical nanoparticle formulation of MK-0869: a beagle dog model predicts improved bioavailability and diminished food effect on absorption in human. *Int. J. Pharm.* **285**:135–146 (2004).
9. B. K. Kang, J. S. Lee, S. K. Chon, S. Y. Jeong, S. H. Yuk, G. Khang, H. B. Lee, and S. H. Cho. Development of self-microemulsifying drug delivery systems (SMEDDS) for oral bioavailability enhancement of simvastatin in beagle dogs. *Int. J. Pharm.* **274**:65–73 (2004).
10. J. B. Dressman. Comparison of canine and human gastrointestinal physiology. *Pharm. Res.* **3**(3):123–131 (1986).
11. J. B. Dressman, and K. Yamada. Animal models for oral drug absorption. In P. G. Welling (ed), *Pharmaceutical Bioequivalence*, Marcel Dekker, New York, 1991, pp. 235–266.
12. K. Yamada, A. Furuya, M. Akimoto, T. Maki, T. Suwa, and H. Ogata. Evaluation of gastrointestinal transit controlled-beagle dog as a suitable animal model for bioavailability testing of sustained-release acetaminophen dosage form. *Int. J. Pharm.* **119**:1–10 (1995).
13. M. Akimoto, N. Nagahata, A. Furuya, K. Fukushima, S. Higuchi, and T. Suwa. Gastric pH profiles of beagle dogs and their use as an alternative to human testing. *Eur. J. Pharm. Biopharm.* **49**:99–102 (2000).
14. C. Y. Lui, G. L. Amidon, R. R. Berardi, D. Fleisher, C. Youngberg, and J. B. Dressman. Comparison of gastrointestinal pH in dogs and humans: implications on the use of the beagle dog as a model for oral absorption in humans. *J. Pharm. Sci.* **75**(3):271–274 (1986).
15. Y. L. He, S. Murby, G. Warhurst, L. Gifford, D. Walker, J. Ayrton, R. Eastmond, and M. Rowland. Species differences in size discrimination in the paracellular pathway reflected by oral bioavailability of poly(ethylene glycol) and D-peptides. *J. Pharm. Sci.* **87**(5):626–633 (1998).
16. W. L. Chiou, H. Y. Jeong, S. M. Chung, and T. C. Wu. Evaluation of using dog as an animal model to study the fraction of oral dose absorbed of 43 drugs in humans. *Pharm. Res.* **17**(2):135–140 (2000).
17. S. S. Davis, E. A. Wilding, and I. R. Wilding. Gastrointestinal transit of a matrix tablet formulation: comparison of canine and human data. *Int. J. Pharm.* **94**:235–238 (1993).
18. M. Akimoto, A. Furuya, T. Maki, K. Yamada, T. Suwa, and H. Ogata. Evaluation of sustained-release granules of chlorphenesin carbamate in dogs and humans. *Int. J. Pharm.* **100**:133–142 (1993).
19. N. Aoyagi, H. Ogata, N. Kaniwa, M. Uchiyama, Y. Yasuda, and Y. Tanioka. Gastric emptying of tablets and granules in humans, dogs, pigs, and stomach-emptying-controlled rabbits. *J. Pharm. Sci.* **81**(12):1170–1174 (1992).
20. J. H. Meyer, J. Dressman, A. Fink, and G. Amidon. Effect of size and density on canine gastric emptying of non-dogestible solids. *Gastroenterology* **89**:805–813 (1985).
21. H. N. E. Stevens, C. G. Wilson, P. G. Welling, M. Bakhshaee, J. S. Binns, A. C. Perkins, M. Frier, E. P. Blackshaw, M. W. Frame, D. J. Nichols, M. J. Humphrey, and S. R. Wicks. Evaluation of Pulsincap™ to provide regional delivery of dofetilide to the human GI tract. *Int. J. Pharm.* **236**:27–34 (2002).
22. I. R. Wilding, S. S. Davis, R. A. Sparrow, J. A. Ziemniak, and D. L. Heald. Pharmacoscintigraphic evaluation of a modified release (Geomatrix®) diltiazem formulation. J. Control. Release **33**:89–97 (1995).
23. K. Kelly, B. O'Mahony, B. Lindsay, T. Jones, T. J. Grattan, A. Rostami-Hodjegan, H. N. E. Stevens, and C. G. Wilson. Comparison of disintegration, gastric emptying, and drug absorption following administration of a new and a conventional paracetamol formulation, using γ scintigraphy. *Pharm. Res.* **20**(10):1668–1673 (2003).
24. B. Abrahamsson, M. Alpsten, B. Bake, A. Larsson, and J. Sjögren. *In-vitro* and *in-vivo* erosion of two different hydrophilic gel matrix tablets. *Eur. J. Pharm. Biopharm.* **46**:69–75 (1998).
25. M. Kamba, Y. Seta, A. Kusai, and K. Nishimura. Evaluation of the mechanical destructive force in the stomach of dog. *Int. J. Pharm.* **228**:209–217 (2001).
26. Y. Iwanaga, J. Wen, M. S. Thollander, L. J. Kost, G. M. Thomforde, R. G. Allen, and S. F. Phillips. Scintigraphic measurement of regional gastrointestinal transit in the dog. *Am. J. Physiol.* **275**:G904–G910 (1998).
27. S. C. Sutton. Companion animal physiology and dosage form performance. *Adv. Drug Del. Rev.* **56**:1383–1398 (2004).
28. J. D. R. Schulze, E. E. Peters, A. W. Vickers, J. S. Staton, M. D. Coffin, G. E. Parsons, and A. W. Basit. Excipient effects on gastrointestinal transit and drug absorption in beagle dogs. *Int. J. Pharm.* **300**:67–75 (2005).
29. B. Abrahamsson, T. Albery, A. Eriksson, I. Gustafsson, and M. Sjöberg. Food effects on tablet disintegration. *Eur. J. Pharm. Sci.* **22**:165–172 (2004).
30. C. S. Cook, L. Zhang, J. Osis, G. L. Schoenhard, and A. Karim. Mechanism of compound- and species-specific food effects of structurally related antiarrhythmic drugs, disopyramide and bidisomide. *Pharm. Res.* **15**(3): 429–433 (1998).
31. B. Abrahamsson, M. Alpsten, B. Bake, U. E. Johnsson, M. Eriksson-Lepkowska, and A. Larsson. Drug absorption from nifedipine hydrophilic matrix extended-release (ER) tablet-comparison with an osmotic pump tablet and effect of food. *J. Control. Release* **52**:301–310 (1998).
32. L. Maggi, L. Segale, E. O. Machiste, A. Faucitano, A. Buttafava, and U. Conte. Polymers–gamma ray interaction. Effects of gamma irradiation on modified release drug delivery systems for oral administration. *Int. J. Pharm.* **269**(2):343–351 (2004).
33. T. Waaler, S. A. Sande, B. W. Müller, and G. Schüller Lisether. The influence of thermal neutron irradiation on the *in-vitro* characteristics of ASA oral dosage forms. Validation of neutron activation. *Eur. J. Pharm. Biopharm.* **43**:159–164 (1997).
34. M. Kamba, Y. Seta, A. Kusai, and K. Nishimura. Comparison of the mechanical destructive force in the small intestine of dog and human. *Int. J. Pharm.* **237**:139–149 (2002).
35. R. A. Hinder, and K. A. Kelly. Canine gastric emptying of solids and liquids. *Am. J. Physiol.* **233**(4):E335–E340 (1977).
36. J. H. Meyer, E. A. Mayer, D. Jehn, Y. Gu, A. S. Fink, and M. Fried. Gastric processing and emptying of fat. *Gastroenterology* **90**:1176–1187 (1986).
37. A. Harrison, A. Betts, K. Fenner, K. Beaumont, A. Edgington, S. Roffey, J. Davis, P. Comby, and P. Morgan. Nonlinear oral pharmacokinetics of the α-antagonist 4-Amino-5-(4-fluorophenyl)-6,7-dimethoxy-2-[4-(morpholinocarbonyl)-perhydro-1,4-diazepin-1-yl]quinoline in humans: use of preclinical data to rationalize clinical observations. *Drug Metab. Dispos.* **32**(2):197–204 (2004).
38. M. Kamba, Y. Seta, A. Kusai, M. Ikeda, and K. Nishimura. A unique dosage form to evaluate the mechanical destructive force in the gastrointestinal tract. *Int. J. Pharm.* **208**:61–70 (2000).
39. N. Fotaki, M. Symillides, and C. Reppas. *In vitro versus* canine data for predicting input profiles of isosorbide-5-mononitrate from oral extended release products on a confidence interval basis. *Eur. J. Pharm. Sci.* **24**:115–122 (2005).
40. M. B. Awang, J. G. Hardy, S. S. Davis, I. R. Wilding, and S. J. Parry. Radiolabeling of pharmaceutical dosage forms by neutron-activation of Sm-152. *J. Labeled Compd. Radiopharm.* **33**(10):941–948 (1993).