Bioequivalence Study of Stressed and Nonstressed Hard Gelatin Capsules Using Amoxicillin as a Drug Marker and Gamma Scintigraphy to Confirm Time and GI Location of *In Vivo* Capsule Rupture

G. A. Digenis,[1,3] E. P. Sandefer,[1] R. C. Page,[1] W. J. Doll,[1,2] T. B. Gold,[1] and N. B. Darwazeh[1]

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Purpose: Evaluate if crosslinked hard gelatin capsules (HGCs) having different *in vitro* dissolution profiles changed *in vivo* release times or altered bioavailability of a drug marker; assess if a two-tier dissolution test (with and without enzyme) predicted *in vivo* performance.

Methods. Two classifications of stressed HGCs were artificially produced by exposure to formaldehyde (HCHO). HGCs were categorized as, a) pass/pass (p/p) which met *in vitro* dissolution criterion (75% drug dissolution at 45 min), b) moderately crosslinked fail/pass (f/p) which failed dissolution criterion in the absence of enzymes and passed in the presence of enzymes, and c) severely crosslinked fail/fail (f/f) which failed *in vitro* standards with or without enzymes. A six-way, single dose bioequivalence study (n = 10) administered the three HGCs under the fasted and fed condition. *In vivo* capsule rupture and GI transit were monitored via gamma scintigraphy, and blood samples were collected through six hours.

Results. Each crosslinked HGC was bioequivalent to the control p/p capsule when using AUC(0–∞) and Cmax for comparison. Mean *in vivo* disintegration of the p/p capsule was 7 ± 5 min for the fasted condition and 11 ± 7 min for the fed condition. *In vivo* rupture for the f/p capsule was 22 ± 12 min and 23 ± 11 min for the fasted and fed studies, respectively, while the f/f HGC ruptured at 31 ± 15 min and 71 ± 19 min under the fasted and fed condition, respectively. Onset of amoxicillin absorption was dependent on *in vivo* HGC rupture and subsequent entry of the released radioactive marker into the small intestine. Consequently, fasted Tmax values were significantly later for the f/p HGC (1.62 ± 0.53 hr) and f/f HGC (1.85 ± 0.58 hr) as compared to the p/p HGC (1.17 ± 0.30 hr). Fed Tmax values were statistically different only for the f/f capsule (2.55 ± 0.44 hr) where Tmax values for the p/p and f/p HGCs under the fed condition were 1.50 ± 0.47 hr and 1.60 ± 0.46 hr, respectively.

Conclusions. A two-tier dissolution procedure that retested a crosslinked hard gelatin capsule with addition of gastric or intestinal enzymes provided an adequate *in vitro* indicator of the formulation's *in vivo* performance. The observed delays in the onset of amoxicillin absorption and Tmax for the severely crosslinked f/f HGC was attributed to delayed *in vivo* capsule rupture, however, this delay did not adversely change AUC(0–∞) nor Cmax.

KEY WORDS: crosslinking; hard gelatin capsule; gamma scintigraphy; bioequivalence; dissolution; enzymes; formaldehyde treatment.

INTRODUCTION

Gelatin capsules, soft and hard, comprise one of the most versatile pharmaceutical oral delivery systems into which drug powders, solutions or suspensions may be filled. The favorable properties of the gelatin capsule includes its strong yet flexible backbone, glossy appearance and ease of swallowing (1). Among the applications that utilize the hard gelatin capsule (HGC), soft elastic gelatin capsule (SEGC) or gelatin coating technology include controlled release formulations, personal care (cosmetic) products, nutritional supplements, and tablet/caplet enrobing with gelatin film.

The proclivity of the gelatin molecules toward formaldehyde-induced derivitization and crosslinking through the ϵ-amino and guanidino functionalities of its lysine and arginine residues has been substantiated by NMR (2–5) and wet chemistry (1,6,7), and has also been considered as a method to produce enteric hard gelatin capsules. More recently, the potential effects of formaldehyde crosslinking in gelatin have been reviewed because of apparent dissolution problems observed in the *in vitro* testing of hard and soft gelatin capsules (1,8). The formation of crosslinks between the tertiary functionalities of the polypeptide backbone in gelatin leads to an increase in the molecular weight of gelatin, and more specifically, a potential detrimental effect on the dissolution of the drug which it encapsulates (5). In a recent study, when HGCs were exposed to atmospheric formaldehyde at concentrations of 150 ppb for as few as two hours, a partial insolubilization of the gelatin shell was observed (9). Namely, the dissolution rate of amoxicillin from stressed capsules was less than the rate of drug dissolution from fresh unstressed capsules (9). Formaldehyde-induced crosslinking manifests itself visibly through formation of a thin, water-insoluble membrane (pellicle) around the gelatin capsule during dissolution testing (8). Being hydrophobic, the pellicle acts as a barrier that restricts the release of a drug.

Recently, much attention has been paid to the source of the low molecular weight aldehydes implicated in gelatin capsule insolubilization. For example, it has been reported that corn starch, a common excipient and fill material in HGCs, may contain low levels of hexamethylenetetramine stabilizer (1,10) which decomposes under humid conditions to give ammonia and formaldehyde. Polyethylene glycols, which serve as solvents for many SEGC pharmaceutical formulations, liberate low molecular weight aldehydes through free radical reactions upon exposure to aerobic conditions (10–12). Furfural, an aldehyde generated in bottles containing rayon coilers, has also been shown to react with gelatin to form a crosslinked insoluble product (13).

Changes in *in vitro* dissolution due to exposure to high heat and/or humidity have been observed in capsules containing chloramphenicol (13,14), tetracycline (10), nitrofurantoin (15) and also water-insoluble or relatively water-soluble agents (16). Because of the observed changes in the *in vitro* dissolution of these products (after exposure to high heat and/or humidity), the principal concern has been to determine the effect of prolonged storage conditions on the bioavailability and/or the clinical efficacy of a drug from gelatin capsules or gelatin-coated tablets. More specifically, an effort has been made in recent years to develop *in vitro* dissolution tests which provide a better indicator of the *in vivo* performance of the stressed gelatin capsule.

[1] University of Kentucky College of Pharmacy, Lexington, Kentucky 40536-0082.

[2] Present address: Scintipharma, Inc., 2201 Regency Rd., Suite 403, Lexington, Kentucky 40503.

[3] To whom correspondence should be addressed. (e-mail: digenis@pop.uky.edu)

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In a series of studies, Mohamad *et al.* showed a decrease in the *in vitro* dissolution rate (in water) of tetracycline hydrochloride from HGCs following storage for 48 months at ambient temperature (10), however, there were no differences in the *in vivo* bioavailability of the antibiotic (17,18). Similar results were also observed by these investigators with HGCs containing ampicillin trihydrate (19). Dey *et al.* showed that bioavailability of etodolac from 300 mg stressed capsules (40°C, 75% RH, 6 months) that had failed specified dissolution criteria were actually found to be bioequivalent to that of control capsules in 24 adult male volunteers (20). Interestingly, the HGCs used in the same study had failed *in vitro* dissolution tests in phosphate buffer (pH 7.5), but met dissolution specifications (not less than 85% of drug dissolved in 30 min) when tested in phosphate buffer containing 1% w/v pancreatin.

The present investigation attempted to further study this phenomenon by artificially producing crosslinked HGCs under controlled laboratory conditions, and determine if an *in vitro* dissolution procedure was predictive of the eventual *in vivo* performance of stressed hard gelatin capsules. Exposure of gelatin capsules to a specific level of formaldehyde with and without heat and humidity produced two different types of stressed capsules (21), which were categorized as moderately or severely crosslinked HGCs. An *in vivo* bioequivalence study was subsequently conducted to determine, through gamma scintigraphy, the time and location in the GI tract of *in vivo* capsule disintegration and compare these *in vivo* parameters to the absorption of amoxicillin. The objectives of this study, in which bioequivalence was referenced to a fresh unstressed capsule group were threefold, 1) compare the *in vivo* time of rupture of the three different capsule types when administered under fasted and fed conditions, 2) examine if a two-tier *in vitro* dissolution test (with and without enzymes) accurately predicted *in vivo* performance of the gelatin capsule, and 3) correlate GI transit with plasma concentration of amoxicillin for each of the three capsule groups to further validate the use of gamma scintigraphy in the evaluation of the *in vivo* behavior of pharmaceutical dosage forms.

MATERIALS AND METHODS

Preparation of Crosslinked Capsule Shells and *In Vitro* Dissolution Criteria

Hard gelatin capsules (Warner-Lambert, Capsugel®, Morris Plaines, NJ; size #1, pink opaque body, royal blue opaque cap) were filled with lactose artificially contaminated with 18 ppm formaldehyde. Crosslinking was initiated by either storing the filled capsules at room temperature or in an atmosphere of high temperature and relative humidity (40°C/75% RH) (21). These storage conditions were maintained for six months after which time the individual capsules were emptied of the formaldehyde-containing lactose powder, and then used within four weeks for *in vivo* assessment. It should also be noted that the procedures used to artificially crosslink HGCs in the current study preceded the crosslinking method recently described (22). These milder conditions (22) were likely to produce a stressed capsule that was not as severely crosslinked as the stressed capsules utilized in the present study. The control capsules were used as received without further treatment.

After exposure to formaldehyde ± heat/humidity, the treated capsule shells were categorized based on USP dissolution criteria for acetaminophen (not less than 75% dissolved at 45 minutes, USP apparatus II, 50 rpm, 900 mL, 37°C, capsules placed in coils). During the initial development of the crosslinking procedures, acetaminophen was originally chosen as a convenient *in vitro* drug marker, thus, all *in vitro* dissolution standards were based on the USP dissolution criterion for acetaminophen (75% dissolution by 45 minutes). Subsequent to the development of the preliminary *in vitro* procedures, a more discriminating drug absorption marker was ultimately chosen for the *in vivo* bioequivalence study. Amoxicillin was selected for the current *in vivo* assessment since this beta-lactam antibiotic has been implicated to capacity-limited absorption by carrier-mediated transport (23), and therefore, it was anticipated that potential differences in bioavailability from the different capsule shells would be more definitive by using a surrogate drug marker like amoxicillin. The treated capsule shells were subsequently filled with amoxicillin (200 mg), and dissolution was completed on the three treatment groups in each of the following four dissolution media, 1) distilled / deionized water adjusted to pH 1.2 (enzyme-free), 2) USP simulated gastric fluid (pH 1.2) with pepsin (1:10,000, 3.2 g/L, Fluka Chemical Corp., Ronkonkoma, NY), 3) phosphate buffer (pH 7.2, enzyme-free) and 4) USP simulated intestinal fluid (pH 7.2) with pancreatin (10 g/L, Aldrich Chemical Co., Milwaukee, WI). Capsules that did not pass the nominal dissolution criterion (75% amoxicillin dissolution by 45 minutes) in the specific dissolution medium were categorized as "fail" (f) capsules while capsules that passed the same dissolution specification were designated as "pass" (p) capsules. Samples were withdrawn at ten minute intervals through 90 minutes. Using the above nomenclature, capsules were categorized as the following:

- untreated control HGCs that passed *in vitro* dissolution tests with and without enzymes (pepsin and pancreatin and referred to as pass/pass or p/p capsules);
- moderately crosslinked HGCs stored at room temperature for six months in the presence of formaldehyde, and failed *in vitro* dissolution tests in the absence of enzyme but passed with addition of enzyme to the dissolution medium (fail/pass or f/p capsules);
- severely crosslinked HGCs stored at elevated heat and humidity for six months in the presence of formaldehyde, and failed *in vitro* dissolution tests in the presence and absence of gastrointestinal enzymes (fail/fail or f/f capsules).

Study Design and Subject Enrollment

Ten healthy male volunteers (24–39 years, mean = 29.3 ± 3.9 yrs) provided informed consent and were enrolled into the study. The research followed the tenets in the Declaration of Helsinki promulgated in 1964, and the protocol was approved by the Institutional Review Board and Radioactive Drug Research Committee at the University of Kentucky (approval received September 1994). A complete medical history and physical examination was given to each volunteer, and fasting blood and urine samples were taken for clinical testing to include urine drug screen, routine blood chemistry and hematology, and testing for hepatitis B surface antigen and HIV.

The study was a balanced, single dose, complete crossover

design involving six treatment conditions that separately administered each of the three capsule types under fasted and fed conditions. Each observation session was separated by three to four days, and all six treatments were administered over a three week period. Volunteers reported to the study unit following a continuous eight hour fast and subjects were required to swallow two radioactive capsules (each containing 200 mg amoxicillin) in either the fasted condition or the fed condition. The standard meal consumed 30 minutes before dose was a high calorie, high fat breakfast (two scrambled eggs, sausage patty, biscuit and 1% milk).

Dosage Form Preparation and Radiolabeling for Gamma Scintigraphy Study

Hard gelatin capsules were treated with formaldehyde (18 ppm) as previously described (21) to obtain the desired *in vitro* release profiles of the drug marker. Fresh amoxicillin capsules (Amoxil®, Smith-Kline Beecham) were emptied of their contents and the capsule shells from each of the three treatment groups (p/p, f/p or f/f capsules) were filled with the equivalent of 200 mg amoxicillin where the drug formulation was radiolabeled with either 25 μCi ^{111}In-DTPA or 50 μCi ^{99m}Tc-DTPA that had been dried onto lactose. The 200 mg fill weight was chosen due to the severe crosslinking of the f/f capsule which resulted in a reduction of the capsule volume, and correspondingly, a decrease in the maximum fill weight.

Dose Administration and *In Vivo* Gamma Scintigraphy

During each treatment period, subjects were administered two capsules (200 mg amoxicillin per capsule) with 240 mL water where one capsule was radiolabeled with ^{111}In-DTPA and the other labeled with ^{99m}Tc-DTPA. The capsules were identical in every way with the exception of the gamma emitting radioisotope used to radiolabel the contents of the capsule. The dual label method permitted independent determination of *in vivo* disintegration of each capsule to increase statistical power and also allowed a measure of intra-subject variability.

Immediately after administration, subjects were positioned supine beneath a gamma scintillation camera (Siemens BasiCam, medium energy collimator, dual label acquisition with a 15% window at 247 keV [^{111}In] and 8% window at 140 keV [^{99m}Tc]). Subjects were imaged continuously for the first 10 to 60 minutes after dosing to accurately determine *in vivo* capsule rupture. Subsequent to this, subjects were scanned for a period of 3–10 minutes at half hour intervals until the last blood sample was withdrawn at six hours post dose. Images were acquired in 1 minute increments and stored on computer generating a time-activity study (Cardiac Medical Systems, Springfield, WI). Venous blood samples (7 mL, sodium heparin) were taken from an indwelling catheter in the arm at 0, 0.33, 0.66, 1, 1.5, 2, 2.5, 3, 4, 5 and 6 hours; plasma was harvested, frozen and sent to an independent laboratory for analysis of amoxicillin using a validated drug assay (reverse phase HPLC with UV detection, Wisconsin Analytical Research Services, Madison, WI).

Pharmacokinetic Analysis and Statistical Comparison

Standard pharmacokinetic analyses to assess bioequivalence were completed to include determination of AUC(0–∞), Cmax, Tmax. Additionally, the time and concentration at onset of amoxicillin absorption were also reported as separate parameters. The AUC(0–∞) values were calculated using the linear trapezoidal rule to the last time point, with extrapolation to infinite time. Statistical comparisons used a repeated measures analysis of variance (RM ANOVA, 90% confidence interval). The two one-sided confidence intervals for Cmax and AUC-(0–∞) were computed using natural log-transformed data (24).

Scintigraphic Analysis and Comparison with Amoxicillin Concentration

Scintigraphic images were analyzed and regions of interest (ROI) were drawn to include the stomach, early small intestine (jejunum), distal small intestine (ileum) and colon (ScinWin™ Scintigraphy Software, PC Solutions, Louisville, KY). Dynamic gastrointestinal (GI) transit curves were generated depicting the quantitative GI transit of radioactivity through these ROI's with the amoxicillin concentration curve overlaid the transit curves. The time and gastrointestinal location of *in vivo* capsule disintegration was also determined and statistical comparisons were made for the two formaldehyde treated capsules versus control (p/p capsule).

RESULTS AND DISCUSSION

In Vitro Dissolution

In vitro dissolution profiles of amoxicillin for each of the three capsule types are shown in Fig. 1. As previously described,

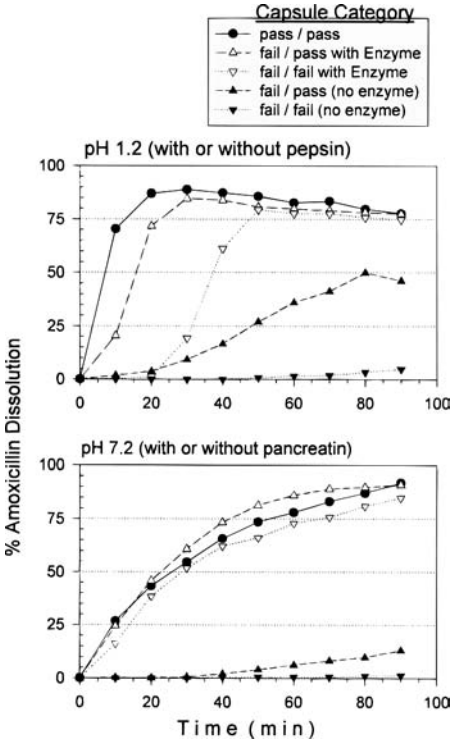

Fig. 1. Mean *in vitro* dissolution of amoxicillin (n = 6) at pH 1.2 and 7.2 (USP paddle, 50 rpm, 37°C) in the presence and absence of enzymes (pepsin [3.2 g/L] or pancreatin [10 g/L]) from non-stressed (p/p) and stressed (f/p and f/f) capsule shells.

acetaminophen was originally used as a convenient *in vitro* drug marker while developing standard laboratory procedures to consistently produce crosslinked hard gelatin capsules. Consequently, the *in vitro* dissolution benchmark used to categorize stressed capsules in the current study was not less than 75% drug dissolution in 45 minutes (specification for acetaminophen, USP 23). Any treated capsule that did not produce 75% drug dissolution by 45 minutes was arbitrarily categorized as a fail capsule for the specific dissolution condition.

As indicated by Fig. 1, the fail/pass capsule that was moderately crosslinked following exposure to formaldehyde (18 ppm, ambient temperature, 6 months) resulted in less than 25% and 5% amoxicillin dissolution at 45 minutes in enzyme free dissolution media at pH 1.2 and 7.2, respectively. The addition of pepsin at pH 1.2 resulted in >75% amoxicillin dissolution by 30 minutes while addition of pancreatin at pH 7.2 required the full 45 minutes to obtain 75% amoxicillin dissolution. The extended time to reach 75% dissolution in the presence of pancreatin for the f/p capsule was not surprising since the inherent rate of amoxicillin dissolution at neutral pH was slower as shown by the control p/p capsule which actually required 50 minutes to achieve the nominal 75% dissolved in the absence of enzyme. Furthermore, qualitative observations made during *in vitro* dissolution tests indicated that the majority of the f/p gelatin shells started to dissolve and release its drug content within five minutes after the capsule was submersed into the dissolution medium containing pancreatin at pH 7.2.

The severely crosslinked fail/fail capsule shell that was produced following exposure to formaldehyde (18 ppm) at elevated heat and humidity (40°C/75% RH) had a different *in vitro* dissolution curve compared to the f/p capsule. Figure 1 demonstrated that the f/f capsule shell at pH 1.2 without enzyme resulted in less than 5% dissolution at 45 minutes and remained relatively unchanged through 90 minutes. Following the addition of pepsin, amoxicillin dissolution from the f/f capsule was faster where approximately 60% was dissolved at 40 minutes and over 75% by 50 minutes. By using the single time point at 45 minutes, the f/f capsule shell nearly met the defined dissolution criterion in the presence of pepsin even though the dissolution curve in its entirety showed a considerable delay in the initial onset of *in vitro* drug release. At pH 7.2, the f/f capsule depicted in the bottom plot of Fig. 1 failed to achieve 75% dissolution by 45 minutes in the presence or absence of pancreatin, however, the nominal dissolution value of 75% was eventually reached at 70 minutes when pancreatin was used (Fig. 1). The relatively high concentration of pepsin (3.2 g/L) and pancreatin (10 g/L) that is specified for simulated gastric fluid and simulated intestinal fluid (as per USP 23) probably contributed to the f/f capsule marginally failing the dissolution standard. Recently, it has been suggested that the concentration and enzyme activity in the dissolution medium can be reduced in order to discern differences between *in vitro* drug dissolution profiles from different HGCs where the ideal pancreatin concentration should not exceed 0.05 g/L and pepsin activity should not exceed 7.5×10^5 units per liter (22). Also note that the slight decline in the amoxicillin dissolution curve at pH 1.2 in Fig. 1 was attributed to acid catalyzed degradation.

Mean Drug Levels and Pharmacokinetics

Mean plasma concentration curves are shown in Fig. 2 for all treatment conditions where the top graph represents the fasted condition and the bottom graph shows the amoxicillin profiles for the fed condition. The mean curves for the fasted condition demonstrated a slight lag in the onset of absorption for the f/p and f/f capsules as compared to the control capsule. Fasted mean amoxicillin concentrations values were statistically different ($p < 0.05$) through 1 hour and 1.5 hours for the f/p and f/f capsules, respectively and are indicated by the cross (+) or asterisk (*). The delays in the onset of *in vivo* absorption from the f/p and f/f capsules were consistent with the observed delays in the *in vitro* dissolution curve at pH 1.2 (with enzyme, top graph of Fig. 1), and underscores the relevance of using the entire *in vitro* dissolution curve to interpret *in vivo* results instead of using only a single time point.

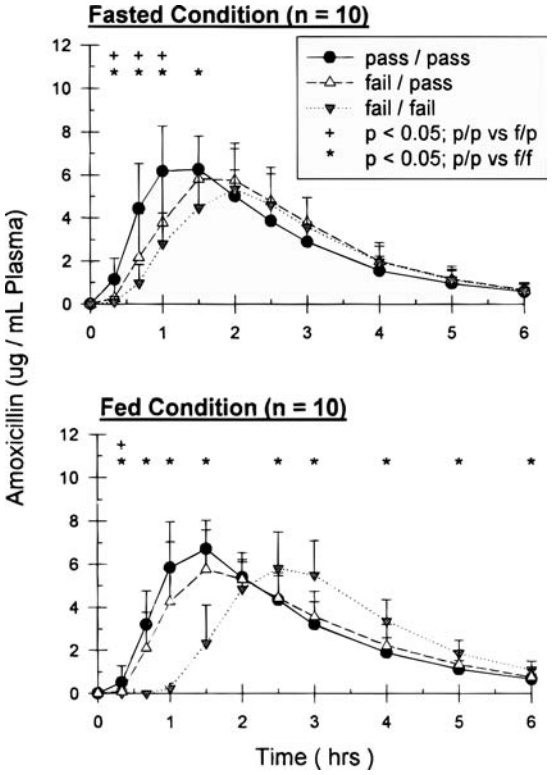

Fig. 2. Mean (± sd) plasma amoxicillin concentration (n = 10) following oral administration of amoxicillin (400 mg; 2 capsules × 200 mg) administered in the fasted and fed condition in non-stressed (p/p) and stressed (f/p and f/f) hard gelatin capsules. Concentrations at time points statistically different from control (p/p) are indicated by a cross (+) for the f/p capsules or asterisk (*) for the f/f capsules.

When administered under the fed condition, the mean curves in the bottom plot of Fig. 2 demonstrated that the delay in amoxicillin absorption was more pronounced for the highly crosslinked f/f capsule, but was less evident for the moderately crosslinked f/p capsule. All blood sampling times for the f/f capsule in the fed condition were statistically different when compared to the p/p capsule except at 2 hrs post dose ($p < 0.05$). The later onset of absorption for the f/f capsule administered in the fed condition was consistent with the use of formaldehyde to crosslink gelatin as a method to make a delayed release enteric capsule. Conversely, only the 0.33 hour time point was different for the f/p capsule under the fed condition (bottom plot, Fig. 2).

Although the initial visual impact of the mean concentration curves in Fig. 2 demonstrated prominent differences at individual time points relative to the control p/p capsule, the actual pharmacokinetic parameters reported in Table I describe less striking differences with regard to AUC(0–∞) and Cmax values. These two parameters can be considered the most relevant measures to assess bioequivalence since most drugs, like amoxicillin, are taken several times a day or dosed to steady state. As indicated in Table I, mean AUC(0–∞) and Cmax values between treatments were not statistically different. This *in vivo* result demonstrates that despite the severe differences in the *in vitro* dissolution curves without enzymes (Fig. 1), the two-tier dissolution test with the addition of enzyme was used successfully to predict bioequivalence even in the worst case scenario of the severely crosslinked f/f capsule. However, Table I also describes that the two, one-sided 90% confidence limits, using natural log-transformed data were outside the 80–125% limits for the following comparisons: 1) AUC(0–∞) for the p/p and f/f HGCs in the fasted condition, 2) Cmax for the p/p versus the f/f capsule in the fasted condition, and 3) Cmax for p/p and f/p HGCs in the fed condition.

Table I. Mean Pharmacokinetic Parameters (n = 10) Following Oral Administration of Amoxicillin (400 mg) in Control Hard Gelatin Capsules (Pass/Pass) and Formaldehyde Treated Crosslinked Hard Gelatin Capsules (Fail/Pass or Fail/Fail Capsules) Under Fasted and Fed Conditions

	FASTED condition			FED condition		
	p/p	f/p	f/f	p/p	f/p	f/f
AUC(0–∞)(μg · h mL^{-1})						
Mean	18.03	18.08	15.93	18.86	18.24	18.56
SD	4.69	4.66	4.70	2.62	3.35	3.42
Cmax (μg/mL)						
Mean	6.78	6.42	5.77	7.02	6.35	6.42
SD	1.96	1.43	2.05	1.24	1.69	1.32
Tmax (hrs)						
Mean	1.17	*1.62	1.85	1.50	1.60	*2.55
SD	0.30	0.53	0.58	0.47	0.46	0.44
$t_{1/2}$ (hrs)						
Mean	1.15	1.19	1.14	1.23	1.21	1.26
SD	0.27	0.22	0.20	0.19	0.20	0.31
Onset of Absorption (hrs)						
Mean	0.36	*0.63	*0.70	0.47	*0.67	*1.20
SD	0.11	0.25	0.19	0.18	0.22	0.26
Concentration at Onset of Absorption (μg/mL)						
Mean	1.32	1.43	1.13	1.42	1.71	*0.61
SD	0.92	1.22	0.64	1.15	1.44	0.46

Two, one-sided 90% confidence limits for natural log-transformed data

Comparison/capsule type and condition	Confidence limit
Ln AUC	
p/p vs f/p, fasted condition	85.3–108.9%
p/p vs f/f, fasted condition	72.7–104.6%
p/p vs f/p, fed condition	91.3–101.1%
p/p vs f/f, fed condition	94.5–101.9%
Ln Cmax	
p/p vs f/p, fasted condition	80.0–113.9%
p/p vs f/f, fasted condition	69.0–100.5%
p/p vs f/p, fed condition	72.2–109.0%
p/p vs f/f, fed condition	81.4–102.6%

Note: Asterisks(*) indicate statistical significance compared to pass/pass capsule for the specific fasted or fed condition ($p < 0.05$,RM ANOVA).

The observed changes in Tmax and the onset of absorption for the f/p and f/f capsules as reported in Table I must be addressed in context to drugs that are taken acutely and not dosed to steady state. As indicated in Table I, Tmax values under the fasted condition for the fail/pass ($Tmax_{fasted}$ = 1.62 ± 0.53 hr) and fail/fail capsule ($Tmax_{fasted}$ = 1.85 ± 0.58 hr) were statistically later ($p < 0.05$) compared to the pass/pass capsule (1.17 ± 0.30 hr). When administered under the fed condition, only the Tmax from the fail/fail capsule was statistically later ($Tmax_{fed}$ = 2.55 ± 0.44 hr) versus the pass/pass capsule ($Tmax_{fed}$ = 1.50 ± 0.47 hr).

The time at which the amoxicillin was first observed in plasma (onset of absorption, Table I) was statistically different compared to reference capsule where the onset of absorption in the fasted condition was 0.63 ± 0.25 h and 0.70 ± 0.19 h for the f/p and f/f capsules, respectively compared to earlier time for the p/p capsule 0.36 ± 0.11 h. When administered under the fed condition, the observed onset of absorption was 0.67 ± 0.22 h and 1.20 ± 0.26 h for the f/p and f/f capsule, respectively compared to the earlier time for the pass/pass capsule (0.47 ± 0.18 h). As previously discussed, the clinical significance of the later onset of drug absorption from a severely crosslinked f/f capsule is critical only when medications are taken for an acute condition (e.g., analgesia) or when the compound must be taken with food where these delays become greater.

Table I also lists the amoxicillin concentration at the onset of drug absorption. This value is important to consider since it can indicate the rate at which the stressed and non-stressed capsules ruptured and released the drug. For example, if the initial concentration for the crosslinked capsule was significantly less than the reference product, then this suggests that the hydrophobic pellicle probably forms an additional barrier to drug dissolution and further postpones or slows the drug absorption phase. There were no statistical differences reported in the fasted condition where the mean concentration at onset of absorption was 1.32 ± 0.92, 1.43 ± 1.22, and 1.13 ± 0.64 μg/mL for the p/p, f/p, and f/f capsules, respectively (Table I). However, a statistical difference was demonstrated in the fed condition between the f/f capsule and the p/p capsule where mean concentrations at the onset of absorption were 0.61 ± 0.46 and 1.42 ± 1.15 μg/mL for the f/f and p/p capsules, respectively. This difference suggests that an additional barrier to drug dissolution may exist in the fed condition. This *in vivo* result can also be explained from observations made during *in vitro* dissolution where the gelatin shell of f/f capsule remained intact in the dissolution medium, and yet, trace levels of amoxicillin were still detected in solution implying that drug release through the intact pellicle might initially involve a diffusion process. As previously discussed, the significance of decreased drug levels during the onset of absorption is most relevant if a drug is prescribed for acute use only.

Individual *In Vivo* Capsule Rupture and Amoxicillin Absorption

Table II summarizes the individual time of *in vivo* capsule rupture where the disintegration of the p/p capsule (7 ± 5

Table II. Time of *In Vivo* Disintegration of Unstressed (Pass/Pass) Capsules, Moderately Cross-linked (Fail/Pass) Capsules, and Severely Cross-linked (Fail/Fail) Capsules Filled with Amoxicillin (200 mg) and Administered Under the Fasted or Fed Condition

	FASTED condition						FED condition					
Subject	p/p		f/p		f/f		p/p		f/p		f/f	
001	5	5	18	23	27	27	10	27	16	18	68	83
002	7	8	35	36	30	68	7	21	12	15	45	52
003	7	8	42	54	10	10	8	9	9	24	52	68
004	7	8	16	16	28	44	5	6	15	15	46	58
005	8	26	17	19	19	19	6	7	21	27	84	95
006	4	6	9	10	27	46	6	8	4	16	93	93
007	5	10	10	19	18	25	23	23	36	36	66	66
008	2	2	18	34	36	66	11	11	23	36	51	113
009	6	9	17	24	24	34	11	11	28	46	68	79
010	7	9	14	14	25	33	7	9	27	27	48	87
Mean	7		*22		*31		11		*23		*71	
SD	5		12		15		7		11		19	
RSD(%)	65.4%		53.5%		50.2%		58.4%		46.7%		27.4%	

Note: Asterisks (*) indicate statistical significance ($p < 0.05$) compared to the pass/pass capsule for the specific fasted or fed condition. Note that each subject was administered two capsules per dosing period, and the *in vivo* disintegration time is listed for each capsule.

min) in the fasted condition was statistically earlier ($p < 0.05$) compared to the f/p capsule (22 ± 12 min) and f/f capsule (31 ± 15 min). When administered under the fed condition, the disintegration time of the p/p capsule (11 ± 7 min) and the f/p capsule (23 ± 11 min) was unchanged relative to the fasted condition, however, the mean *in vivo* disintegration time of the f/f capsule (71 ± 19 min) was more than doubled when compared to fasted condition.

Figures 3 and 4 represent individual amoxicillin curves for all subjects in the fasted and fed condition, respectively. These two figures also list the GI locus of *in vivo* capsule rupture, and the individual times of *in vivo* capsule disintegration are reproduced from Table II for easy cross-referencing. Figure 3 indicates that in the fasted condition, the severely crosslinked f/f capsule emptied intact from the stomach in seven subjects to release amoxicillin in either the proximal small intestine (jejunum) or distal small intestine (ileum). The f/p capsule was observed to empty the stomach intact under the fasted condition in only three subjects (005, 006 and 009). Conversely, when administered under the fed condition, Fig. 4 shows that the presence of food resulted in all capsules rupturing and delivering their drug content in the stomach.

The individual amoxicillin profiles represented in Figs. 3 and 4 were consistent with the mean amoxicillin profiles where the onset of absorption and Tmax from the crosslinked f/p and f/f capsules were delayed compared to the p/p capsule in most subjects. Analysis of the scintigraphic data indicated that amoxicillin absorption was very rapid once the capsule ruptured and the released radioactive marker entered the small intestine, thus, any delay in absorption was directly related to the later capsule rupture for the f/p and f/f capsule.

As indicated by Table II and Figs. 3 and 4, *in vivo* capsule rupture was most significantly delayed when the f/f capsule was administered thirty minutes after food where the mean time of capsule rupture was more than doubled as compared to the f/f capsule taken under the fasted condition. The reason for this significant increase was probably due to the severity of crosslinking in the fail/fail capsule and resulted in a drug concentration profile having the appearance of an enteric coated product.

To further understand the factors that influenced *in vivo* disintegration times of crosslinked capsules and its effect on systemic drug levels, it is necessary to reconsider the chemistry of gelatin crosslinking. Stressing of gelatin with formaldehyde results in increased crosslinking of its linear polypeptide strands which increases its molecular weight, and changes rigidity and swelling characteristics. Furthermore, crosslinked gelatin exhibits decreased aqueous solubility and number of its free (underivatized) lysine and arginine residues. The above chemical changes result in a decrease in the extent and rate of proteolysis of the gelatin due to the decreased availability of its peptide bonds toward proteolytic enzymes, like pepsin and pancreatin. These enzymes cease to recognize carboxyl ends of the formaldehyde-derivatized lysine and arginine. This is further exacerbated by a decrease in rate and penetration by proteolytic enzymes due to the hydrophobic character of the crosslinked sites on the surface of gelatin. Specifically, the methylene lysine-lysine or lysine-arginine methylene crosslinks, formed by the formaldehyde-induced stressing of gelatin (2–5), appear to be less sensitive to degradation by pepsin because of the inaccessibility of the enzyme's active site and the hydrophobic character of the crosslinked gelatin (5).

Detailed analysis of the time and location of *in vivo* capsule disintegration suggested that the formaldehyde produced crosslink was more susceptible to cleavage by pancreatic enzymes in the small intestine as compared to gastric enzymes like pepsin. For example, under the fasted condition, the mean time of capsule rupture for fail/fail capsules when drug release occurred in the stomach was 40 ± 18 minutes ($n = 8$ capsules), whereas the mean time of capsule rupture for fail/fail capsules that ruptured in the small intestine was less (24 ± 10 minutes, $n = 12$ capsules). Furthermore, if the time of gastric residence is subtracted from the preceding value, then the mean lag time that the intact f/f capsules resided in the small intestine after

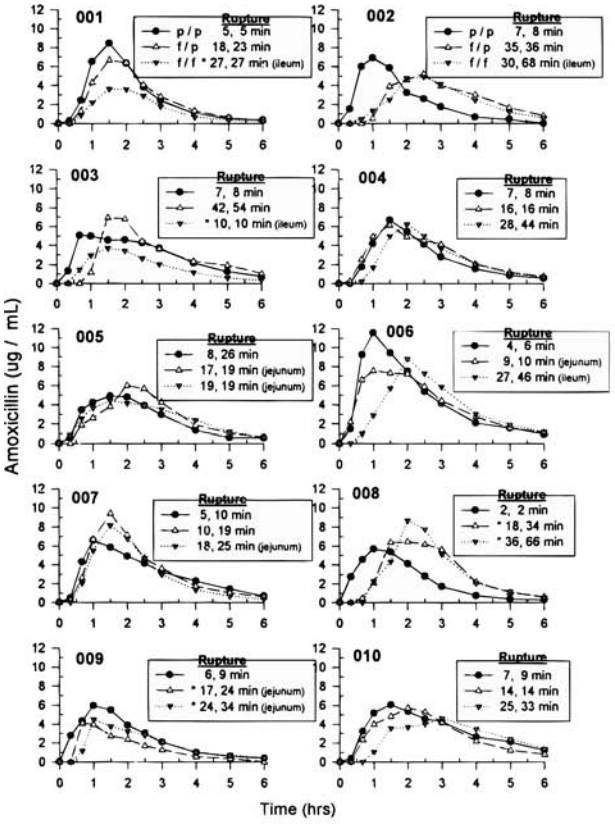

Fig. 3. Fasted subjects (001–010) administered 400 mg amoxicillin (2 capsules × 200 mg each). The individual time of *in vivo* capsule rupture is indicated for each dose. Subjects who did fall within individual 80:120 for AUC(0–∞), compared to the p/p capsule are noted by an asterisk. All capsules ruptured in the stomach unless otherwise noted in parenthesis in the legends.

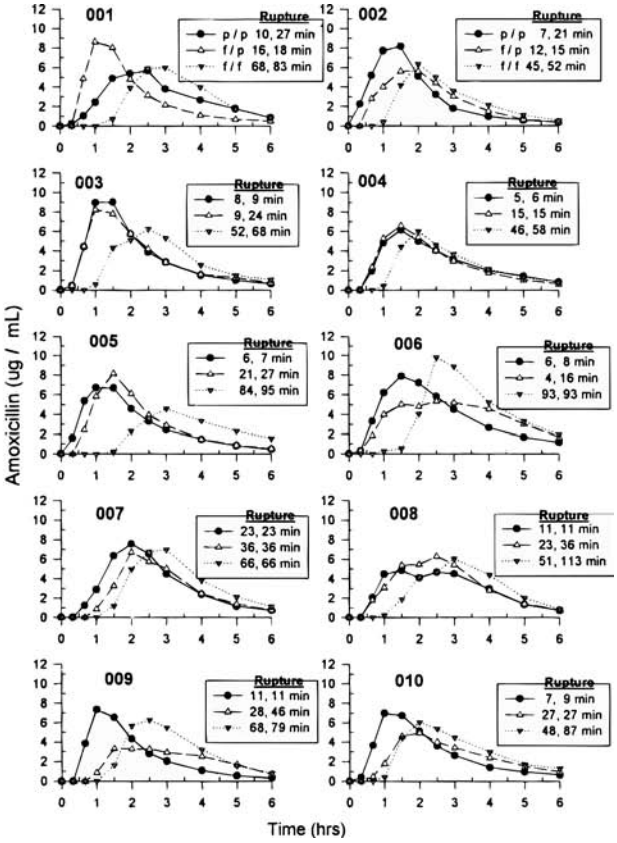

Fig. 4. FED subjects (001–010) administered 400 mg amoxicillin (2 capsules × 200 mg each). Individual time of *in vivo* capsule rupture is indicated within each legend. All capsules ruptured in the stomach under the fed condition.

gastric emptying was only 10 ± 8 minutes. The mechanical forces associated with gastric emptying and transit of the intact capsule in the small intestine may have also contributed to the apparently faster capsule rupture in the small intestine.

The disintegration times of the fail/pass capsules in the fasted condition can also be treated in a similar manner, thus, when release occurred in the stomach, the time of capsule disintegration was 26 ± 12 minutes (n = 15 capsules). Fail/pass capsules that emptied from the stomach intact resulted in a mean *in vivo* disintegration time of 16 ± 6 minutes (n = 5 capsules), and after subtracting gastric residence, the actual time that these five fail/pass capsules resided in the small intestine was only 4 ± 1 minutes until rupture was observed. These data collectively support the probability that intestinal enzymes, like pancreatin, may be more efficient in breaking the crosslink functionality. This observation is also supported by the *in vitro* dissolution curves shown in Fig. 1 where initial amoxicillin dissolution from the severely crosslinked f/f capsule was fastest at pH 7.2 in the presence of pancreatin whereas at pH 1.2 with pepsin, significant amoxicillin dissolution (>10%) did not occur until 30 minutes for the f/f capsule. These results further emphasize the added value that the two-tier dissolution test offered for predicting *in vivo* results.

Intra-Subject Bioequivalence

It is also of interest to consider the bioequivalence of the different capsule types in the individual subjects. Figure 5 provides a rapid visual comparison of individual bioequivalence where ratios of untransformed AUC(0–∞) and Cmax values are plotted for all treatment conditions relative to the control. Using the boundary set by the 80:120 rule for untransformed data, Fig. 5 displays that the majority of individual dosings fell within the 80:120 margin for AUC(0–∞) while individual Cmax resulted in greater variability relative to the control treatment.

Figure 5 indicates four instances where the individual AUC(0–∞) ratio was less than 0.8. The severely crosslinked f/f capsule administered under the fasted condition represented three cases (subjects 001, 003, and 009), and the fourth case where the AUC(0–∞) ratio fell below the 80:120 margin occurred in subject 009 from the moderately crosslinked f/p capsule in the fasted condition. The reason for the reduced extent of absorption was readily apparent from the gastrointestinal transit data as determined by gamma scintigraphy. It was observed in these four circumstances that the crosslinked capsules emptied the fasted stomach intact and rapidly moved to the distal jejunum or ileum where the capsules eventually ruptured. At first inclination, the reduction in the extent of amoxicillin absorption can be interpreted as being caused by the intact capsule bypassing the duodenum and the majority of the jejunum where amoxicillin was expected to be most efficiently absorbed (23). However, if this was an accurate explanation, then amoxicillin absorption would have been less in the other subjects who emptied the stressed capsules intact from the fasted stomach. In fact, in the six remaining instances where

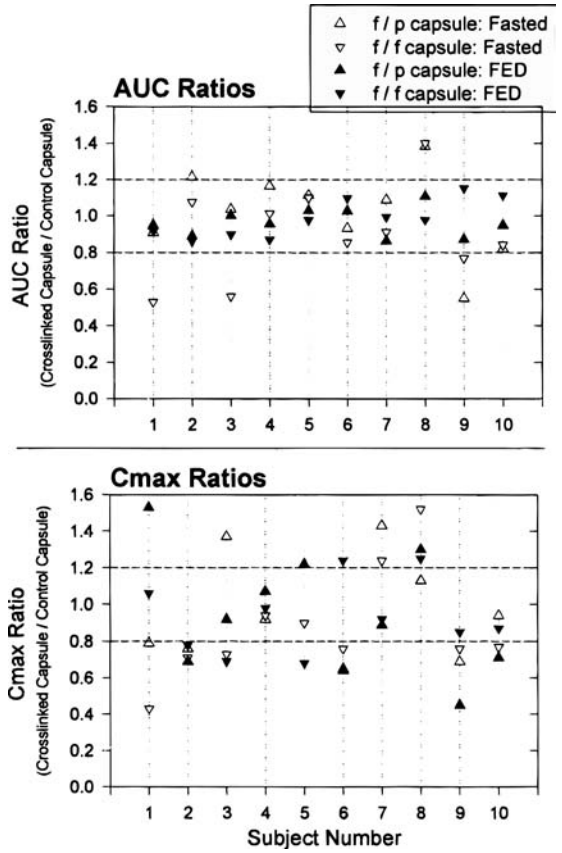

Fig. 5. AUC(0–∞) and Cmax ratios relative to control p/p capsules in individual subjects following oral administration of 400 mg amoxicillin (2 capsules × 200 mg) administered in stressed capsules (f/p and f/f).

crosslinked capsules were observed to empty the fasted stomach intact (e.g., f/f capsule for subjects 002, 005, 006 and 007, and f/p capsule for subjects 005 and 006, Fig. 3), the AUC(0–∞) ratio for these subjects was within the 80:120 limit (refer to Fig. 5).

Comprehensive analysis of the GI transit data revealed that the most probable cause for reduced amoxicillin absorption was due to a decrease in the total small bowel residence after capsule rupture had occurred. Specifically, in the four instances where the AUC(0–∞) ratio was less than 0.80 (Fig. 5), the arrival of the radioactive marker in the colon started within one to two hours post dose. Conversely, in the six other instances where the capsule emptied from the stomach intact and resulted in no change in the extent of drug absorption, the arrival of the radioactive marker at the colon occurred later (three to five hours post dose). It is probable that the longer small bowel residence contributed to improving the extent of amoxicillin absorption in these cases, and furthermore, amoxicillin was not the discriminating drug marker for the upper small intestine as was originally anticipated based on our interpretation of existing literature which described carrier-mediated absorption of amoxicillin (23).

The possibility that total small bowel residence influenced the extent of amoxicillin absorption was further supported by subject 008 where the AUC(0–∞) ratio actually exceeded 1.20 for the f/p and f/f capsules under the fasted condition (Fig. 5). In this particular subject, the control treatment resulted in colonic arrival that was unusually early (two hours post dose) and resulted in an AUC(0–∞) value for the p/p capsule that was atypically low. Colonic arrival for the f/p and f/f capsules for subject 008 occurred at four to five hours post dose, and consequently, the extent of amoxicillin absorption was greater and resulted in an individual AUC(0–∞) ratio that exceeded 1.20. These collective findings are also consistent with positive correlations previously reported between small bowel residence and the extent of drug absorption for other compounds including an enteric multiparticulate of erythromycin (25–27) and an effervescent formulation of ranitidine (28,29).

As previously described, the bottom plot in Fig. 5 indicated that intra-subject variations in Cmax ratios exceeded the 80:120 boundary more frequently than the AUC(0–∞) ratios. The reasons for deviation from the 80:120 range with regard to individual Cmax can be attributed to several related variables including, 1) the time and gastrointestinal location for each of the two capsules to rupture since *in vivo* release of the two co-administered capsules did not always occur at the same time, 2) the rate at which solubilized drug was introduced to region(s) of drug absorption which was dependent on physiologic processes like gastric emptying or the intrinsic rate of amoxicillin solubility at acidic or neutral pH, and 3) timing of transport processes to movement of other components in the GI tract (e.g., food, bile salts) which may facilitate or compete against the drug absorption process.

Although the individual variations observed in Cmax were not important for the clinical efficacy of the drug marker used in the current study, it is possible that significant clinical effects may occur for drugs that have narrow therapeutic indices where pharmacologic efficacy may not be reached when Cmax is low, or conversely, adverse events may be experienced when Cmax is too high. With regard to these issues of safety and efficacy, the intestinal permeability of a drug can become a critical parameter in predicting *in vivo* absorption *a priori* from crosslinked capsules. For example, it is conceivable that highly soluble and highly permeable drugs (Class I of the biopharmaceutic classification system (30)) can result in a high Cmax when released directly in the small intestine due to a rapid and concentrated drug input directly at the site of absorption.

Recently, it was reported that the Cmax of acetaminophen was increased when administered in a f/f capsule under the fasted condition as compared to a p/p capsule (31). Although gamma scintigraphy was not used to verify the site of drug release in the study (31), it is probable that the f/f capsule emptied intact from the stomach in some of the subjects to deliver acetaminophen as a bolus in the small intestine once capsule rupture occurred. Because of the high solubility and permeability of acetaminophen, a rapid absorption phase of acetaminophen was likely to have resulted in a higher Cmax relative to the immediate release p/p capsule where the absorption rate was primarily dependent on the gastric emptying rate since drug release would intuitively have occurred in the stomach for the p/p capsule. Conversely in the current study using amoxicillin, a probable reason that Cmax was not consistently increased with the f/f capsule under the fasted condition was due to the slower rate of amoxicillin dissolution at neutral pH as compared to a faster dissolution rate at acidic pH (refer to Fig. 1). Consequently, in those instances where the f/f capsule was emptied from the stomach intact, the amoxicillin absorption rate was limited by the rate of amoxicillin dissolution in the neutral pH of the small intestine. These results also demonstrate

the potential that gamma scintigraphy has in helping to define and categorize the biopharmaceutical classification of drugs.

In Vitro/In Vivo Comparisons and Pharmacoscintigraphy

One of the primary objectives of this investigation was to determine if a two-tier *in vitro* dissolution test (with and without enzyme) was meaningful towards predicting the eventual *in vivo* performance of crosslinked hard gelatin capsules. The slight delay but complete *in vitro* drug dissolution that was observed when enzymes were added to the dissolution medium shown in Fig. 1 were used to accurately predict the observed *in vivo* delay in the onset of amoxicillin absorption, later Tmax, but equal AUC(0±`) and Cmax values relative to the control p/p capsule. Furthermore, the *in vitro* dissolution profiles in Fig. 1 suggested that the crosslink functionality showed greater susceptibility to pancreatin compared to pepsin which was also demonstrated *in vivo* as previously described. Had the second tier dissolution test not added enzyme to the dissolution medium, then the resulting *in vitro* dissolution profiles without enzyme would erroneously predict that both the moderately and severely crosslinked hard gelatin capsules would end in bioinequivalence and product failure (Fig. 1). Therefore, it is conclusive that conventional USP dissolution procedures without enzyme were unable to accurately reproduce the environment in the human gastrointestinal tract nor were these traditional tests useful in providing a meaningful *in vitro/in vivo* comparisons for crosslinked hard gelatin capsules. Consequently, it is apparent that a two-tier dissolution test employing gastric or intestinal enzymes is a valid method to recertify a specific drug batch if it is suspected that gelatin crosslinking or pellicle formation has occurred. The data from the current study indicated that the second dissolution test with enzyme provided an *in vitro* procedure that had greater physiologic relevance with regard to the capsule's eventual *in vivo* behavior.

To underscore the relative accuracy that the two-tier *in vitro* dissolution test offered towards predicting *in vivo* drug release, representative scintigraphic images for subject 005 in the fed condition are shown in Fig. 6 for the p/p, f/p and f/f capsules while the three corresponding quantitative GI transit/amoxicillin concentration overlay plots are shown in Fig. 7. As shown by the images in Fig. 6, disintegration of the p/p capsule was rapid as displayed by the representative image at 0.33 hr. The corresponding overlay plot for the p/p capsule is shown by the top plot of Fig. 7 and describes in detail that the two p/p capsules ruptured at 6 and 7 minutes after dosing and gastric emptying was initiated at approximately the same time (left y-axis). Detailed evaluation of the top plot in Fig. 7 and images like those shown in the left column of Fig. 6 indicated that at 0.33 hr some of the radioactive marker remained near the pylorus of the stomach while the majority (. 80%) had entered the jejunum. Gastrointestinal transit like this typically results in a rapid onset of amoxicillin absorption as demonstrated in the top plot of Fig. 7 (0.33 h for the p/p capsule) where the absorption phase continued through 1.0 to 1.5 h as the radioactive marker moved through the jejunum. The amoxicillin elimination phase started at 1.5 h post dose when approximately 90% of the radioactive marker reached the early ileum.

The middle column of Fig. 6 illustrates the scintigraphic images for the moderately crosslinked f/p capsule in subject 005 in the fed condition. In general, the gastrointestinal transit

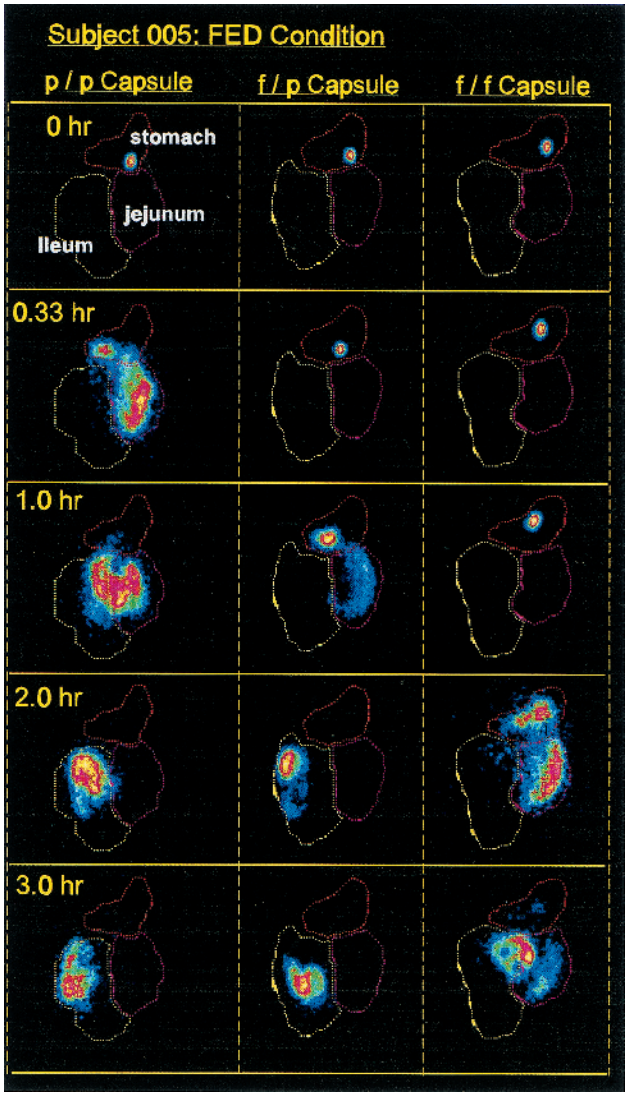

Fig. 6. Representative scintigraphic images for subject 005 administered p/p, f/p and f/f capsules under the fed condition. Regions of interest depicting the early and distal small intestine approximate the location of the jejunum and ileum, respectively. Corresponding overlay plots are shown in Fig. 7.

events for the f/p capsule in the fed condition were initiated twenty to forty minutes later than the control p/p capsule. The image recorded at 0.33 hrs indicated that the f/p was still intact in the antrum of the stomach while at one hour post dose, the f/p capsule had clearly ruptured with a concentrated portion of radioactivity remaining near the pylorus while a significant amount of the surrogate marker was also dispersed through the early small intestine. The middle overlay plot for the f/p capsule depicted in Fig. 7 illustrates capsule rupture at 21 and 27 minutes post dose while gastric emptying started shortly thereafter as confirmed by the onset of amoxicillin absorption by the next blood sample drawn at 40 minutes. Because of the slightly later *in vivo* capsule rupture and gastric emptying times, the amoxicillin concentration profile for the f/p capsule was shifted towards the right (see Figs. 4 and 7). However, the rate of amoxicillin absorption was relatively the same for the p/p and f/p capsule due to similar gastric emptying rates (and therefore

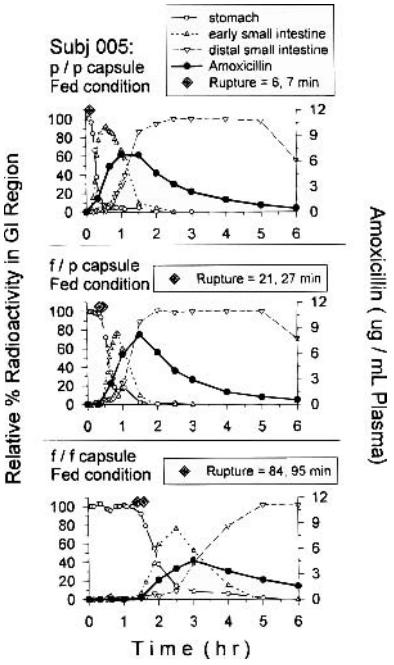

Fig. 7. Overlay plots demonstrating dynamic gastrointestinal transit curves with corresponding amoxicillin profiles for subject 005 after oral administration of the p/p, f/p and f/f capsules under the fed condition (2 capsules × 200 mg amoxicillin). Corresponding representative scintigraphic images are shown in Fig. 6.

similar rates of entry into the small intestine where amoxicillin was rapidly absorbed).

Conversely, the results from the f/f capsule shown by the scintigraphic images in the far right column of Fig. 6 and quantitatively expressed by the bottom overlay plot in Fig. 7 depict different GI transit profiles compared to the control and moderately crosslinked capsules. In general, the *in vivo* behavior of the f/f capsule was likened to a large enteric coated dosage form with the exception that drug release eventually occurred in the stomach under the fed condition. The intact f/f capsule was too large to pass through the constricted pylorus of the fed stomach, and therefore, it was retained until the crosslinked gelatin shell softened enough to initiate drug release. In the example shown in Figs. 6 and 7, this most severely crosslinked capsule was observed to remain intact in the fed stomach through one hour where the two administered f/f capsules ultimately ruptured at 84 and 95 minutes. Initial gastric emptying was relatively slow, and consequently, the ensuing rate of amoxicillin absorption was also slower as compared to the other treatments where in trace levels of amoxicillin were detected at 1.5 h. The Tmax for the f/f capsule in subject 005 was observed at 3.0 h when the stomach was nearly empty of the radioactive marker. These results were anticipated based on the results of the two-tier dissolution test shown in Fig. 1.

CONCLUSIONS

To date, there has been no instance of testing formaldehyde induced crosslinked capsules with definitive *in vivo* testing that has correlated *in vivo* capsule performance in the GI tract with drug absorption (32). Consequently, this study attempted to evaluate critical parameters by using amoxicillin as a surrogate drug marker and gamma scintigraphy to evaluate the time and GI locus of *in vivo* capsule rupture.

It is well known that increased times of exposure to formaldehyde, heat and humidity lead to increased crosslinking of gelatin, and the rate of proteolysis of gelatin by pepsin and/or pancreatin will decrease according to the degree of crosslinking. The results of this study demonstrated that for moderately crosslinked capsules (f/p capsules), a two-tier dissolution test with addition of enzyme was predictive of its *in vivo* performance, and furthermore, implementation of the two-tier test can potentially minimize the number of *in vivo* bioequivalence studies needed to maintain a product on the market if crosslinking problems are suspected. The results of this study also demonstrated that even in the most extreme case of encountering a highly crosslinked capsule, the f/f scenario was still shown to be bioequivalent with regard to amoxcillin AUC(0–∞) and Cmax values even though both *in vitro* dissolution procedures tests failed. Thus, these results suggest that in the remote possibility that periodic testing reveals the existence of a f/f capsule shell, it is still possible that subsequent *in vivo* tests will still show bioequivalence.

Although the preceding conclusions are positive and encouraging, it must nonetheless be recognized that the highly crosslinked gelatin capsules were shown to exhibit *in vivo* behavior similar to that of enteric coated single unit dosage forms. When administered under fed conditions, a significant delay in the onset of amoxicillin absorption and Tmax was observed, and under fasted conditions, the f/f capsules emptied from the stomach intact and released their drug content directly in the small intestine. It is therefore still possible that these scenarios of *in vivo* dosage form behavior may have adverse effects on the bioavailability or bioequivalence of some drugs if, 1) there is preferential absorption in the proximal portion of the small intestine; 2) the capsule shell contains a delayed released enteric coated multiparticulate where drug release will occur even further down the small intestine and may result in incomplete drug absorption and 3) delays in Tmax and onset of absorption can have a significant effect for drugs that are taken acutely and not dosed to steady state.

Regardless of the outcome of the second dissolution test with enzyme, *in vitro* dissolution problems encountered with immediate release gelatin products should be considered on a case by case basis. It is conservatively advised that these occurrences need to be evaluated for each individual drug taking into account the compound's absorption characteristics from the GI tract, intestinal permeability, dosing regimen, pharmacologic effect and safety profile before reapproving a specific drug supply based on the second tier *in vitro* test when enzymes are implemented.

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