The Effect of Gelatin Cross-Linking on the Bioequivalence of Hard and Soft Gelatin Acetaminophen Capsules

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Purpose. To determine if changes in the *in vitro* dissolution of hard and soft gelatin acetaminophen capsules, which result from gelatin crosslinking, are predictive of changes in the bioavailability of the capsules in humans.

Methods. Both hard and soft gelatin capsules were "stressed" by a controlled exposure to formaldehyde, resulting in unstressed, moderately stressed and highly stressed capsules. *In vitro* dissolution studies were conducted using water or SGF with and without pepsin as the media. Separate 24-subject, 3-way crossover human bioequivalence studies were performed on the unstressed and stressed acetaminophen capsules. Plasma acetaminophen was determined by high performance liquid chromatography (HPLC) for 12 hr after each dose.

Results. The *in vitro* rate of dissolution of hard and soft gelatin capsules was decreased by crosslinking. The bioequivalence studies showed that both hard and soft gelatin capsules, which failed to meet the USP dissolution specification in water, but complied when tested in SGF containing pepsin, were bioequivalent to the unstressed control capsules. The capsules that were cross-linked to the greatest extent were not bioequivalent to the unstressed control capsules, based on Cmax. A trend toward an increase in Cmax with increased level of crosslinking was observed, but this was only significant for the severely stressed capsules.

Conclusions. On the basis of this study a two-tier *in vitro* dissolution test was developed using enzymes to distinguish between bioequivalent and bioinequivalent gelatin capsules.

KEY WORDS: Gelatin capsules; acetaminophen; crosslinking; dissolution; human bioequivalence.

INTRODUCTION

When gelatin is exposed to high humidity, elevated temperature or aldehydes, the gelatin may become crosslinked (or stressed), rendering the gelatin resistant to dissolution in water. Digenis *et al.* (1) have reviewed the pharmaceutical implications of the crosslinking of gelatin capsules. Dey *et al.* (2) have investigated the dissolution of etodolac in hard gelatin capsules exposed to high relative humidity and temperature. They also studied the stressed and unstressed capsules in bioavailability studies using dogs and humans. Others (3) have studied changes in the *in vitro* dissolution of soft gelatin capsules containing acetaminophen or nifedipine. While changes in the dissolution of gelatin capsules with time are well known, correlations between the *in vitro* dissolution and *in vivo* bioavailability of stressed capsules have not been studied in detail. Further, the use of an aqueous dissolution media, without protease enzymes such as pepsin or pancreatin, may not reflect the dissolution of gelatin capsules *in vivo* where such enzymes exist in the fluids of the gastrointestinal tract. Acetaminophen was selected as a model drug because it is a common drug that is very safe in moderate doses, and it can be easily quantitated in plasma by HPLC and it is well absorbed from the human intestinal tract after oral administration (4).

EXPERIMENTAL

Dosage Forms

Three lots of hard gelatin capsules were prepared: unstressed, moderately stressed and highly stressed (5). Lactose was exposed to formaldehyde, and the formaldehyde treated lactose was subsequently diluted with pure lactose to achieve formaldehyde levels of 20 ppm and 120 ppm. The diluted lactose samples were filled by hand into size 1 natural transparent gelatin capsules. The capsules containing 20 ppm of formaldehyde were stored at room temperature for six days. The capsules containing 120 ppm of formaldehyde were stored for one day at 40°C and 75% relative humidity (RH), followed by six days at room temperature. The 20 ppm and 120 ppm capsules were then emptied, and the unstressed and stressed capsules were hand-filled with 280 mg of acetaminophen. The *in vitro* dissolution of the capsules was determined after the capsules had been prepared, just before the bioequivalence study was conducted and after the bioequivalence study was completed.

Three lots of soft gelatin capsules were prepared by adding 0, 20 ppm and 80 ppm of formaldehyde to the capsule fill that was composed of 250 mg of acetaminophen in a polyethylene glycol base, which was a mixture of PEG 600 and 1000. The capsule was Type B gelatin, 150 bloom limed-bone gelatin, with glycerin and sorbitol as plasticizers. *In vitro* dissolution studies were carried out over a 30-week period to evaluate the storage conditions required for the capsules to be used in the bioequivalence study. The capsules were stored at 40°C and 75% RH, or 25°C and 60% RH (6), during the crosslinking process. The capsules were stored at ambient temperature after preparation.

Dissolution

In vitro dissolution was carried out with the USP Apparatus 2, at 50 rpm, in 900 ml of 37°C water or simulated gastric fluid

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(SGF) with and without pepsin 1:10,000. The pepsin concentration was 3.2 g/L, and the pepsin had an activity of 885 Units/mg on a hemoglobin substrate.

Bioequivalence Studies

Two separate 24-subject, 3-way crossover, bioequivalence studies with identical protocols were performed for the three lots of the hard gelatin capsules and the three lots of the soft gelatin capsules, with different degrees of stressing and different dissolution profiles. The hard gelatin capsule bioequivalence study used the capsules described above. The soft gelatin capsule bioequivalence study used the unstressed capsules, and the 80 ppm stressed capsules stored for 55 days at 40°C and 75% RH, or 52 weeks at 25°C and 60% RH. The research followed the tenets of the Declaration of Helsinki promulgated in 1964, and was approved by the University's Institutional Review Board and the Risk Involving Human Subjects Committee of the FDA. All subjects provided written informed consent. All subjects were evaluated with a medical history, physical exam, clinical chemistry, complete blood count, urinalysis and ECG prior to entering the study, and were within 10% of ideal body weight. The study designs employed six sequences, with four subjects randomly assigned to each sequence. A washout period of two days was used between each dose. All doses were administered after an overnight fast, along with 180 ml of room temperature water. No food was permitted until a standard lunch was served five hours after dosing. A standard dinner was also served ten hours after dosing. Seven milliliter blood samples were obtained by venipuncture or through an indwelling venous catheter into heparinized evacuated tubes just prior to the dose and at 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, 3, 4, 6, 8, 10 and 12 hr after each dose. Plasma was removed by centrifugation at 4°C and the plasma was stored in glass vials at −20°C until analyzed for acetaminophen by HPLC. The hard gelatin study used six female and 18 male subjects, ranging in age from 21–32 years. The soft gelatin study used seven female and 17 male subjects ranging in age from 21–33 years.

Plasma Analysis

Acetaminophen concentrations were determined by HPLC after extraction of 0.25 ml of plasma with 2 ml of ethyl acetate. A 0.25 ml aliquot of 2-acetamidophenol (0.2 mg/ml) or theophylline (0.2 mg/ml) was added to the plasma samples as an internal standard for the hard and soft gelatin plasma samples, respectively. Evaporated plasma extracts were reconstituted with 150 μl of 20% methanol in water. Plasma standards were prepared from drug-free human plasma fortified with 0.25 ml aliquots of acetaminophen in water, to yield a standard curve over a range of 0.1 to 10.0 μg/ml. Quality control fortified plasma samples were also prepared to contain 0.19, 4.8 and 9.6 μg/ml of acetaminophen. A standard curve in duplicate and the controls in triplicate were assayed each day that subject samples were analyzed. The HPLC system (Waters Associates) consisted of a M6000 pump (1.2 ml/min); a WISP 710B autosampler (40 μl injection); a variable wavelength detector (248 nm); and a Waters Symmetry C-8 column (150 cm × 3.9 mm). The mobile phase contained 14% methanol in 0.05M phosphate buffer (6 g anhydrous monosodium phosphate/liter). The run time between injections was 15 min.

Pharmacokinetic and Statistical Analysis

The maximum plasma concentration (Cmax), time to reach the maximum concentration (Tmax), and the lag-time (Tlag) were determined by inspection of the data. The Tlag was defined as the elapsed time from the time of dosing until the last sampling time before a measurable concentration of acetaminophen appeared in the blood. The area under the plasma concentration-time curve (AUC) was computed using the linear trapezoidal rule. The AUC to infinite time (AUC(0–∞)) was calculated using standard methods (7). Cmax and AUC(0–∞) were also normalized for the mg/kg dose given to each subject. In addition, oral clearance (CI) was calculated as Dose/AUC(0–∞), with and without normalization for body weight. The statistical analysis was performed using the GLM procedure from the SAS statistical package on a VAX 8000 computer. For the model, the main effects were Week, Subject, Drug and Subject within Sequence. The two, one-sided 90% confidence intervals were computed for Cmax and AUC(0–∞) using log-transformed data (8). A one-way ANOVA was utilized to determine gender effects.

RESULTS AND DISCUSSION

Assay

The standard curves exhibited good linearity ($r^2 \geq 0.99$). The precision for the assay of the standards and controls was also good, with relative standard deviations of 4–14% and 6–15% for the standards and controls, respectively.

Dissolution

The results of the dissolution testing are summarized in Table I, and are similar to results presented in detail elsewhere

Table I. *In Vitro* Dissolution Testing of Hard and Soft Gelatin Capsules[a]

Capsule type/media[b]	Mean (SD) percent dissolved (N = 12)				
	15 min	30 min	45 min	60 min	75 min
Soft Gelatin					
P/P Water	84 (6)	98 (1)	98 (1)	99 (1)	99 (2)
F/P Water	5 (1)	31 (9)	72 (13)	101 (2)	101 (2)
F/F Water	1 (1)	8 (4)	21 (6)	45 (16)	69 (14)
F/P SGF + Pepsin[c]	41 (24)	89 (5)	95 (2)	97 (2)	97 (2)
F/F SGF + Pepsin[a,c]	3 (1)	28 (3)	70 (13)	98 (1)	98 (1)
Hard Gelatin					
P/P Water	41 (8)	80 (7)	98 (3)		
F/P Water	15 (6)	37 (10)	55 (13)		
F/F Water	0 (0)	0 (1)	1 (1)		
P/P SGF	45 (10)	81 (11)	99 (6)		
F/P SGF	16 (6)	36 (12)	52 (14)		
F/F SGF	0 (0)	0 (0)	1 (1)		
P/P SGF + Pepsin[c]	48 (10)	82 (11)	96 (7)		
F/P SGF + Pepsin[c]	51 (9)	83 (9)	98 (5)		
F/F SGF + Pepsin[c]	1 (3)	7 (4)	34 (10)		

[a] N = 6.
[b] P/P = ≥75% in water; F/P = ≤75% in water, but ≤75% in SFG with pepsin; and F/F = ≤75% in both water and SGF with pepsin.
[c] Pepsin 3.2 g/l, Activity 1:10,000 Product No. P7000, Sigma Chemical Company, St. Louis, MO.

Table II. Gender Comparisons

	Hard gelatin capsules[a]					Soft gelatin capsules[a]				
	N = 54		N = 18			N = 50		N = 20		
Parameter	Males mean	(M) CV%	Females mean	(F) CV%	F/M ratio	Males mean	(M) CV%	Females mean	(F) CV%	F/M ratio
Dose (mg/kg)	3.59	10	4.16	10	1.16	3.18	12	3.77	12	1.19
Cmax (μg/ml)	4.43	41	3.71	18	0.84	3.90	30	4.87	29	1.25[b]
Cmax/(mg/kg)	1.22	37	0.89	18	0.73[b]	1.20	25	1.27	28	1.06
AUC (0–∞) (μg.hr/ml)	13.3	26	13.1	19	0.99	11.9	21	15.3	20	1.29[b]
AUC (0–∞)/(mg/kg)	3.67	25	3.12	15	0.85[b]	3.69	19	4.01	20	1.09
T1/2 (hr)	2.86	22	2.39	14	0.83[b]	3.03	20	2.67	19	0.88[b]
Tmax (hr)	0.96	66	1.31	44	1.37[b]	0.78	62	0.78	45	1.00
Tlag (hr)	0.24	128	0.32	93	1.33	0.21	114	0.40	102	1.94[b]
Oral Cl (L/hr)	22.4	24	22.1	19	0.99	22.1	24	17.0	23	0.77[b]
Oral Cl/kg	0.29	22	0.33	18	1.14[b]	0.28	21	0.26	20	0.92

[a] Means include unstressed, moderately stressed, and highly stressed capsules.
[b] $p < 0.01$.

(9). The USP 23 has a dissolution specification for acetaminophen capsules of ≥75% dissolved in 45 min, using Apparatus 2, at 50 rpm and 900 ml of water. The unstressed hard and soft gelatin capsules both met this specification with water or SGF with pepsin and were designated pass/pass (P/P). The hard gelatin capsules that were prepared with 20 ppm of formaldehyde failed the dissolution specification in water, but passed in SGF with pepsin. This lot was designated fail/pass (F/P). The hard gelatin capsules that were prepared with 120 ppm of formaldehyde failed dissolution with both media, and were designated fail/fail (F/F). The F/F capsules were tested before and after the bioequivalence study and were found to range in dissolution from 35–41% dissolved at 45 min. The soft gelatin capsules that contained 20 ppm of formaldehyde in the fill were not sufficiently cross-linked, and exhibited the same dissolution profiles in water as the unstressed (P/P) soft gelatin capsules. Thus the 20 ppm lot was not used in the bioequivalence study. The 80 ppm soft gelatin capsule lot stored at 40°C and 75% RH for 55 days failed to meet the USP specifications with water, but passed with SGF and pepsin as the media (F/P). The dissolution of the F/P soft gelatin capsules in water remained at 72–73% at 45 min for the first through the last day of the bioequivalence study. The 80 ppm capsules stored at 25°C and 60% RH for 52 weeks failed to meet USP requirements using either water or SGF with pepsin (F/F). These data demonstrate that gelatin crosslinking can delay dissolution *in vitro*.

Bioequivalence Study

The 24 subjects in each study successfully completed the study, and there were no reports of any adverse reactions other than mild headaches. The data for one male and one female in the soft gelatin capsule study was not included in the bioequivalence statistical analysis because of an interferring peak in the chromatography.

Gender

The results of the gender analysis for both studies is summarized in Table II. The only parameter that was significantly different in both studies was T1/2, with a 12–17% shorter half life in the females. Even in those female/male comparisons that showed significance, the actual differences were relatively small.

Area Under the Curve

The primary bioequivalence parameters are summarized in Tables III and IV for the hard and soft gelatin capsules, respectively. There was very little difference in the AUC(0–∞) values among the three lots of each capsule type, likely because acetaminophen is well absorbed in the intestine (4). Thus a delayed release of drug from the dosage form would not be expected to have a significant effect on the extent of absorption for the highly permeable, highly soluble acetaminophen, even for the capsules that were severely cross-linked. The 90% confidence limits, based on Ln transformed AUC(0–∞) data are summarized in Table V. These data show that the F/P and F/F stressed capsules were bioequivalent to the P/P capsules, based

Table III. Summary of Mean Bioavailability Parameters for the Hard Gelatin Capsule

Parameter	Capsule designation	Mean (CV%)	Ratio[a]	Statistical significance[b]
Cmax (μg/ml)	P/P	3.85 (32)	1.00	ns
	F/P	4.29 (36)	1.11	ns
	F/F	4.60 (44)	1.19	$p = 0.075$
AUC(0–∞)	P/P	12.91 (25)	1.00	ns
(μg.hr/ml)	F/P	13.50 (25)	1.05	ns
	F/F	13.28 (25)	1.03	ns
Tmax (hr)	P/P	0.93 (51)		ns
	F/P	0.99 (73)		ns
	F/F	1.23 (54)		ns
Tlag (hr)	P/P	0.08 (170)		ns
	F/P	0.16 (73)		ns
	F/F	0.55 (65)		$p < 0.001$

[a] Relative to P/P.
[b] ns: $p > 0.05$.

Table IV. Summary of Mean Bioavailability Parameters for the Soft Gelatin Capsule

Parameter	Capsule designation	Mean (CV%)	Ratio[a]	Statistical significance[b]
Cmax (μg/ml)	P/P	3.92 (30)	1.00	ns
	F/P	4.16 (35)	1.06	ns
	F/F	4.40 (31)	1.12	ns
AUC(0–∞)	P/P	12.83 (25)	1.00	ns
(μg.hr/ml)	F/P	12.57 (26)	0.98	ns
	F/F	12.87 (22)	1.00	ns
Tmax (hr)	P/P	0.67 (45)		ns
	F/P	0.78 (67)		ns
	F/F	0.85 (57)		ns
Tlag (hr)	P/P	0.10 (145)		ns
	F/P	0.16 (71)		ns
	F/F	0.52 (67)		$p < 0.001$

[a] Relative to P/P.
[b] ns: $p > 0.05$.

on AUC(0-∞), since the confidence limits were well within the FDA acceptance criteria of 80–125%.

Maximum Plasma Concentration, Tmax and Tlag

Differences were seen among the various capsule lots in the parameters that are affected by rate of release, i.e., Cmax, Tmax and Tlag, as shown in Tables III and IV. The mean Cmax values were higher with an increase in the degree of stressing, although the differences among the three lots of each type of capsule were not statistically significant ($p > 0.05$). The Cmax for 17 of the 24 subjects (71%) who received the hard gelatin capsules was greater for the F/F capsules than for the P/P capsules. In the soft gelatin capsule study the Cmax was greater for the F/F capsules compared to the P/P capsules for 12 of the 22 subjects (55%). The 90% confidence limits for Cmax exceeded the FDA 80–125% limit for the F/F versus the P/P hard and soft gelatin capsules. Thus the F/F and P/P capsules were not bioequivalent based on Cmax, as shown in Table V. The mean Tmax values also showed an increase with increased stressing, but the differences were not significant ($p > 0.05$). However, for Tlag the difference of 25–28 min seen when comparing the P/P and F/F lots of the hard and soft gelatin capsules did reach statistical significance ($p < 0.001$). These data indicate that the release of the drug was delayed until gastrointestinal enzymes were able to breakdown the cross-linked gelatin capsule, and the delay was related to the degree to which the capsule was stressed. In further support of this observation it was determined that the number of subjects without measurable plasma acetaminophen concentrations at 0.25 hr and 0.5 hr was the greatest for the F/F capsules, as shown in Fig. 1.

A dosage form with a longer Tmax will often exhibit a lower Cmax because of the slower release of the drug. In the case of the stressed capsules, an increase in Tmax paralleled an increase in Cmax. Gastric emptying has been reported to be the rate-limiting-step in the gastrointestinal absorption of acetaminophen (10). These observations suggest that the unstressed and moderately stressed capsules began to release drug earlier than the highly stressed capsules, and a larger fraction of the dose in the unstressed and moderately stressed capsules was in solution in the stomach. Gastric emptying of an acetaminophen solution has been described as an exponential process (11). Thus the amount of drug reaching the intestine

Table V. Two, One-Sided 90% Confidence Interval for Log Transformed Data

Comparison	Confidence interval (%)	
	Hard gelatin capsules	Soft gelatin capsules
Cmax		
F/P vs P/P	98–124	93–117
F/F vs P/P	104–132	100–126
AUC(0–∞)		
F/P vs P/P	100–109	95–102
F/F vs P/P	99–106	98–106

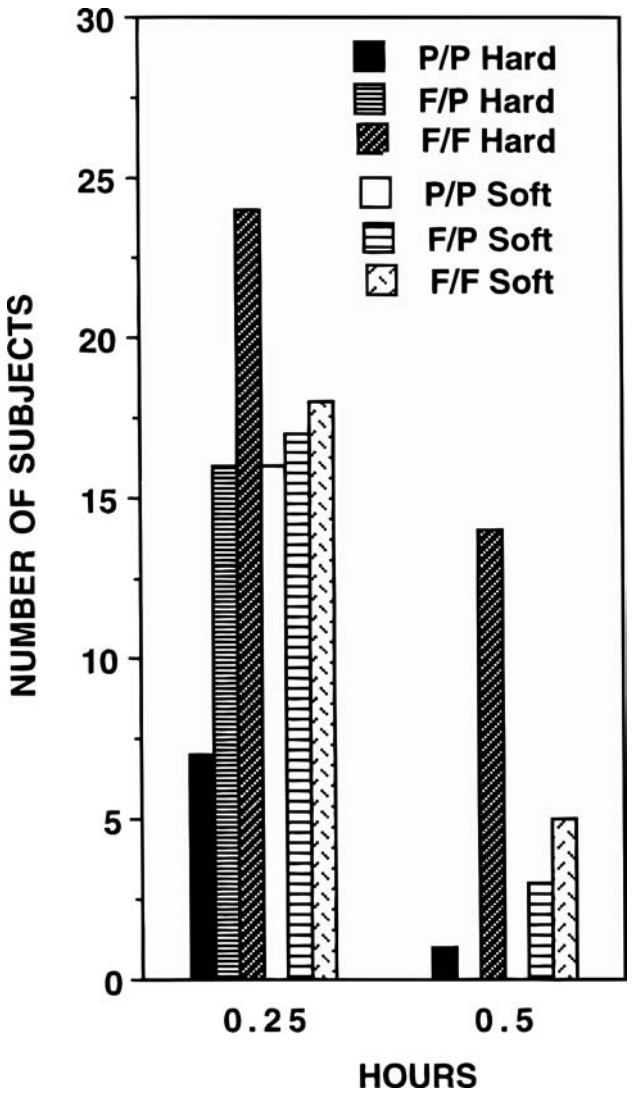

Fig. 1. Number of subjects with no measurable acetaminophen plasma concentrations at 0.25 hr and 0.5 hr after dosing with the P/P. F/P and F/F hard and soft gelatin capsules.

at any given time could be smaller for the unstressed and moderately stressed capsule than for the highly stressed capsule that may release a greater fraction of the dose all at once after gastric emptying. The results of this study are consistent with the results of a scintigraphic study that demonstrated that there was no significant difference in the *in vivo* disintegration of unstressed and moderately stressed hard gelatin capsules containing acetaminophen (12).

Dissolution Specifications

Based in part on the results of these studies, the USP has revised the dissolution specifications for gelatin capsule dosage forms, and now permits a two-tier procedure. If the gelatin dosage form fails to pass the dissolution specification given in the USP, a second test is utilized with a dissolution media that contains either SGF with pepsin or simulated intestinal fluid with pancreatin, depending on the dissolution media specified for the product, and the solubility of the drug (9,13). In the present study capsules that failed both tiers of the dissolution test were not bioequivalent to the unstressed control capsules, while capsules that passed both tiers were shown to be bioequivalent.

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