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Use of Enzymes in the Dissolution Testing of Gelatin Capsules and Gelatin-Coated Tablets—Revisions to Dissolution <711> and Disintegration and Dissolution of Dietary Supplements <2040>

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ABSTRACT

A revision to the general chapters Dissolution <711> and Disintegration and Dissolution of Dietary Supplements <2040> is being proposed to address the shortcomings of the current chapters regarding the use of enzymes in the dissolution test of gelatin capsules or gelatin-coated tablets. The recommendations are to use the enzymes pepsin (medium with pH equal to or below 4.0), papain or bromelain (medium with pH above 4.0 and below 6.8), and pancreatin (medium with pH equal to or above 6.8) if the dosage form does not comply to the appropriate *Acceptance Table* or, in the case of dietary supplements, appropriate *Tolerances* requirements due to the presence of cross-linking in the gelatin. Also, the revision clarifies how the enzyme activity should be determined, the amount of enzymes to be added to the medium, and a pre-treatment procedure to be used when the dissolution medium contains surfactant or any other ingredient known or suspect to inactivate the enzyme being used. This *Stimuli* article provides the rationale for these revisions and presents data to support the recommendations being made. Readers are encouraged to send any comments, questions, suggestions or data to the corresponding author. Also, see the section *Other Related Revisions* at the end of this article for information on other related general chapters and monographs.

INTRODUCTION

The USP general chapters Dissolution <711> (*1*) and Disintegration and Dissolution of Dietary Supplements <2040> (*2*) allow the addition of enzymes to the dissolution medium when hard or soft gelatin capsules and gelatin-coated tablets do not conform to the dissolution specification.

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The current text in these chapters is:

For hard or soft gelatin capsules and gelatin-coated tablets that do not conform to the Dissolution specification, repeat the test as follows. Where water or a medium with a pH of less than 6.8 is specified as the Medium in the individual monograph, the same Medium specified may be used with the addition of purified pepsin that results in an activity of 750,000 Units or less per 1000 mL. For media with a pH of 6.8 or greater, pancreatin can be added to produce not more than 1750 USP Units of protease activity per 1000 mL.

These instructions present some challenges:

1) The text "that do not conform to the Dissolution specification" is open for interpretation because it does not exclusively relate the dissolution failure to the presence of cross-linking in the gelatin. The user can assume that the enzymes can be used for any type of failure, even those not related to gelatin cross-linking. Also, there is no clear definition of what constitutes the Dissolution

specification.

2) The chapter recommends the use of pepsin when the medium is water or it has a pH less than 6.8. The pH for optimal activity of pepsin is up to a pH of 4; pepsin has almost no protease activity above pH 5.5 (*3*).

3) The use of purified pepsin is recommended. The specification for this enzyme is in the *Reagent Specifications* section of *USP–NF* (*4*). Not all users are aware that this specification contains the procedure for determining the appropriate pepsin activity. Commonly, the amount of pepsin to be added to the medium is based on the information displayed on the reagent label or in the certificate of analysis, where the units used may not be comparable or equivalent to those obtained when following the procedure in the purified pepsin specification in *USP–NF*.

4) Pepsin has good activity up to pH 4 and pancreatin is to be used for dissolution media with pH above 6.8, there is no current recommendation for an enzyme appropriate for the pH range from 4 to 6.8.

5) The text does not give any guidance regarding dissolution media that contain surfactant or other ingredients that may denature the enzyme being used.

A revision to <711> and <2040> is being published in 40(6) issue of *Pharmacopeial Forum* to address these issues and make recommendations for the appropriate procedures to be followed. This *Stimuli* article gives the scientific background for these revisions and present data to support the recommendations being made.

CROSS–LINKING IN GELATIN CAPSULES

One of the major factors that can affect the properties of the gelatin capsule shell is the chemical interactions between the fill material and gelatin or between the gelatin and environment during storage, which can result in gelatin cross-linking. Cross-linking entails the formation of strong chemical linkages beyond simple hydrogen and ionic bonding between gelatin chains (*5–7*). One of the strongest and most common types of cross-linking involves the covalent bonding of the amine group of a lysine side chain of one gelatin molecule to a similar amine group on another gelatin molecule. This reaction generally is catalyzed by trace amounts of reactive aldehydes. Formaldehyde, glutaraldehyde, glyoxal, and reducing sugars are the most common catalysts. The covalent bonding produced with this type of cross-linking is, for all practical purposes, irreversible, and will eventually render the gelatin insoluble. When the gelatin is no longer soluble in water, dissolution of the shell must involve the breaking of other bonds, e.g., by enzyme-mediated breaking of peptide bonds in protein chains. Common causes of cross-linking include:

1) Aldehydes present in active pharmaceutical ingredients, excipients, packaging materials, or degradants formed *in situ* during storage (e.g., polyethylene glycols that may auto-oxidize to form aldehydes)

2) High humidity

3) Substances that facilitate a cross-linking reaction

4) Substances that promote decomposition of stabilizers used in excipients such as hexamethylenetetramine in corn starch resulting in the formation of ammonia and formaldehyde

5) Rayon coilers that contain an aldehyde functional group (furfural)

6) Polyethyelene glycols that may auto-oxidize forming aldehydes

7) UV light, especially in the presence of high temperature and humidity

8) Heat, which can catalyze aldehyde formation

Cross-linking typically results in the formation of a pellicle on the internal or external surface of the gelatin capsule shell that prevents the capsule fill from being released. Cross-linking is evidenced by the observation of a thin membrane or gelatinous mass during dissolution testing (*8*).

In vitro dissolution testing of cross-linked capsules can result in slower or incomplete release of the active ingredient or no release at all (*9, 10*). The degree of cross-linking is not usually uniform within one capsule or among different capsules. As consequence, dissolution results will have higher variability when gelatin capsules are cross-linked (*5, 6, 8, 9, 11*).

EVIDENCE OF CROSS-LINKING

The easiest way to confirm dissolution failure resulting from cross-linking is by visual observations. The capsule is going to hydrate and swell but it is not going to rupture. A thin membrane or gelatinous mass can be seen around the capsule (see Figures 1 and 2).

Several techniques can be used to determine the nature and extent of gelatin cross-linking, including carbon 13-nuclear magnetic resonance, ultraviolet and fluorescence spectrophotometry, magnetic resonance imaging (*12*), Fourier transform near-infrared spectroscopy (*12, 13*), and others.

Another option to identify gelatin cross-linking during method development is to do a capsule switching test, when possible. This can be done by transferring the content of the cross-linked capsules into fresh capsules and the dissolution test is run again (*14*).

HISTORY OF THE USE OF ENZYMES IN DISSOLUTION TESTING

In the early 1990s, a Gelatin Capsule Working Group was formed and charged with the task of assessing the issue of non-compliance of the in vitro dissolution results and its potential impact on the bioavailability of gelatin capsules drug products. This group was composed of members of pharmaceutical industry trade associations, gelatin capsule manufacturers, USP, academia, and several offices within the U.S. Food and Drug Adminis-
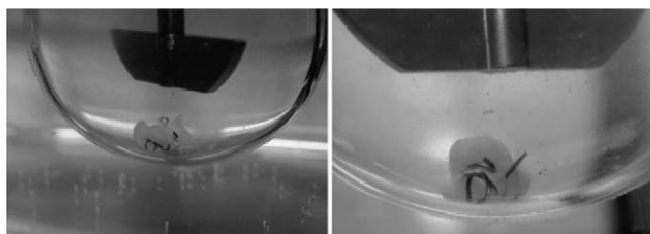
Figure 1. Cross-linked gelatin capsule with sinker in USP Dissolution Apparatus 2. ©2014 Vivian Gray. Used with permission.

Figure 2. Cross-linked gelatin capsule in USP Dissolution Apparatus 1. ©2014 Vivian Gray. Used with permission.

tration (FDA). The information gathered by this group indicated that satisfactory dissolution rates might be obtained for bioavailable products upon the addition of pepsin or pancreatin enzymes to the dissolution medium. The Working Group developed a protocol to use carefully stressed hard and soft gelatin capsules to determine the relationship of the in vitro to the in vivo performance. These stressed capsules were compared to unstressed capsules in two bioequivalence studies. The completed bioequivalence studies indicated that moderately stressed gelatin capsules, which do not meet the standard dissolution test specifications but do pass the test upon the addition of enzymes to the medium, are bioequivalent to unstressed capsules. Over-stressed capsules, which fail to meet the dissolution test specifications with and without enzymes in the medium, failed to demonstrate bioequivalence. In vitro studies were conducted using pepsin in simulated gastric fluid and water, and pancreatin was used in simulated intestinal fluid at pH 6.8 and 7.5. On the basis of the results of these studies, the Working Group recommended that a second step or tier be added to the standard USP or approved dissolution test. This two-step test was found appropriate for all gelatin capsules and gelatin coated tablets at any time, including at the batch release of a marketed product (15, 16). The results of these studies were used to establish the amount of pepsin and pancreatin that could be added to the dissolution medium in the case of test failure due to the presence of cross-linking in the gelatin. The two-steps dissolution testing was included in the *First Supplement of USP 24* (17).

ENZYMES IN DISSOLUTION TESTING

The current version of <711> (1) and <2040> (2) recommends the use of pepsin for dissolution media with pH values less than 6.8 and pancreatin for dissolution media with pH values equal to or greater than 6.8. Because pepsin has good protease activity in pH values up to 4 (3), there is a need to select appropriate proteases that could be used with dissolution media that have pH in the range from 4 to 6.8. Papain and bromelain were identified as potential candidates for this application. Their use is being recommended only as proteases to digest cross-linked
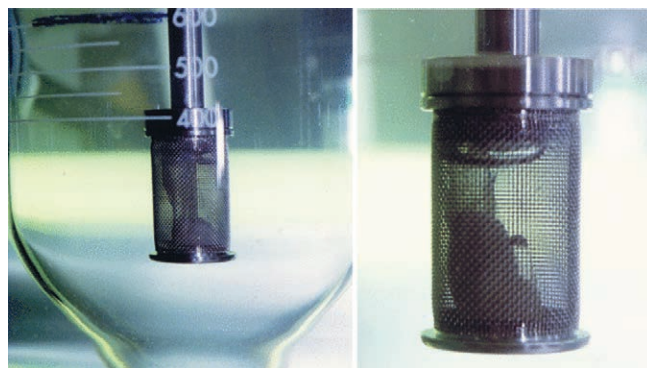
gelatin in dissolution media; they are not simulating any in-vivo condition.

Pepsin

Pepsin is obtained from the glandular layer of hog stomach (18). It can also be obtained from cattle or sheep (19). Its typical applications include: preparation of fishmeal and other protein hydrolysates and in the clotting of milk to manufacture cheese (18). Other applications include: dietary supplements, digestive aids, protein hydrolysis, leather tanning, silver recovery from films, dissolution testing, and others.

Figure 3 shows the relationship between pH and activity and stability of pepsin. Pepsin is stable over the pH range of 1–6 (3). Above pH 6, pepsin is rapidly irreversibly inactivated. At pH 8 pepsin is completely inactivated (3, 20). Pepsin has a maximum activity at pH 2, 70% of the maximal peptic activity is still present at pH 4.5, and almost no
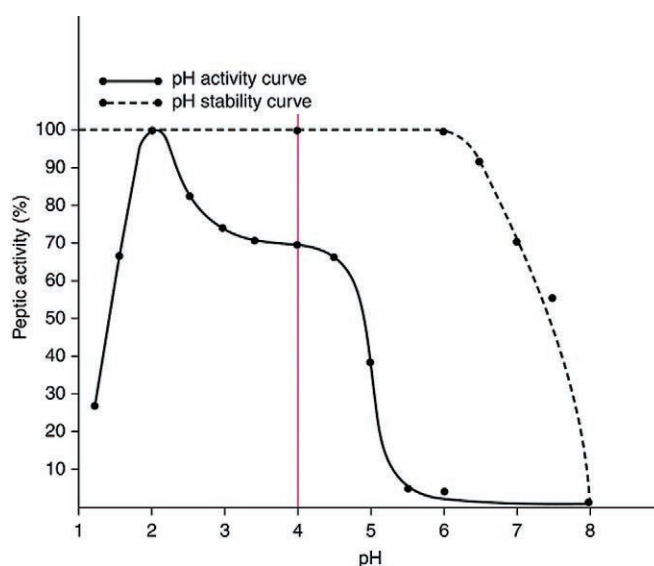

Figure 3. The pH stability and pH activity of pepsin (3).

peptic activity at pH 5.5 (*3, 21*). Figure 3 also shows that although pepsin is inactive at pH 6.5, more than 90% of the pepsin is still stable. The inactive pepsin at pH 6.5 can be reactivated when the pH is lowered to 2.0, retaining 70% of its original activity. Pepsin remains stable at pH 7.0 for at least 24 hours at 37 °C, retaining around 79% of its original activity after re-acidification to pH 2 (*22*). Pepsin as dry powder is stable for 3 years at room temperature and for extended periods of time when stored at –10 °C to –25 °C.

Pepsin activity can be determined using different substrates: egg albumen (*23*), hemoglobin (*4, 18, 19*), milk (*24*). Recently, USP revised the procedure that uses hemoglobin to make it as similar as possible to the procedure in the *Food Chemical Codex* (FCC) (*18*). The revisions were published in *Pharmacopeial Forum* (*PF*) (*25, 26*) with the option of using a calibration curve or a single point. Several comments and suggestions were received and the revised hemoglobin procedure will be published in a future issue of PF. With the implementation of the FCC procedure for the activity determination of pepsin, the amount currently recommended in *USP* of 750,000 Units/L will be revised and it will expressed as USP units/L.

Papain

Papain is a proteolytic enzyme obtained from the latex of the green unripe fruit of the papaya (*Carica papaya*) (*27, 28*). Papain is typically sourced from tropical areas, including India and the Democratic Republic of Congo. Papain applications include: dietary supplement, meat tenderizer, treatment for insect bites, cell culture, cell isolation, chill-proofing (clarifying) beer, protein hydrolysis, and others. Papain will digest most protein substrates more extensively than the pancreatic proteases.

Although papain aqueous solutions have good thermal stability, the solution stability is pH dependent. Papain solutions are unstable under acidic conditions, i.e., at pH values below 2.8, there is a significant loss in activity (*27*). Typically, the optimal pH for papain activity is 6.0–7.0 but it varies according to the substrate, the optimal pH is about 5 for gelatin and about 7 for casein and egg albumen (*29*).

The proteolytic activity of papain is most commonly determined using casein substrate (*27, 30*). Other substrates that can be used are hemoglobin (*31*), milk (*32*), egg albumen, and gelatin (*33*).

Papain, as a dry powder, is stable for 2 years when stored at 2–8 °C, retaining 90–100% of its potency. It has a markedly shorter shelf life at room temperature, retaining 70–80% of its potency when stored for 2 years at room temperature.

Bromelain

Bromelain is a proteolytic enzyme obtained from stems of pineapple (*Ananas comosus*) (*34–36*). Bromelain is

sourced from tropical areas including Philippines, Brazil, Indonesia, and Thailand. Its typical applications include: dietary supplements (anti-inflammatory action, platelet aggregation, etc.), meat tenderizer, leather tanning, protein hydrolysis, protein stain remover, chill-proofing (clarifying) beer, and lowering the protein level of flour in baking applications (*37–39*).

The optimum pH value is influenced by the nature of the substrate, the concentration and type of buffer, and the presence of reducing agents (*37*). The most common range is pH 5 to 7, but bromelain can also function in the pH range of 3 to 9. Bromelain is stable over the pH range of 3.0 to 6.5 and, once it has combined with its substrate, the activity is no longer susceptible to the effect of the pH (*38*). Figure 4 shows the effect of pH on the activities of native bromelain (NB) and two types of chemically modified bromelain (PB and PMB), which indicated that the optimum pH values were 7.0, 8.0, and 9.0, respectively (*40*).

Bromelain as a dry powder is stable for 1.5 to 3 years when stored at 2–8 °C. If stored at temperatures higher than 25 °C, the activity should be retested at 3 months intervals. In aqueous solution, bromelain rapidly deteriorates through self-digestion. The addition of serum containing α_2-macroglobulin will prevent self-digestion (*39*).

Bromelain is commonly assayed using a gelatin substrate but it can also be assayed using casein, hemoglobin, and milk (*34, 36–38*).

Pancreatin

Pancreatin is an enzyme complex containing enzymes with various substrates specificities. These enzymes include trypsin, α-chymotrypsin, carboxypeptidase, lipase, and amylase. It is produced by exocrine cells of the pan-
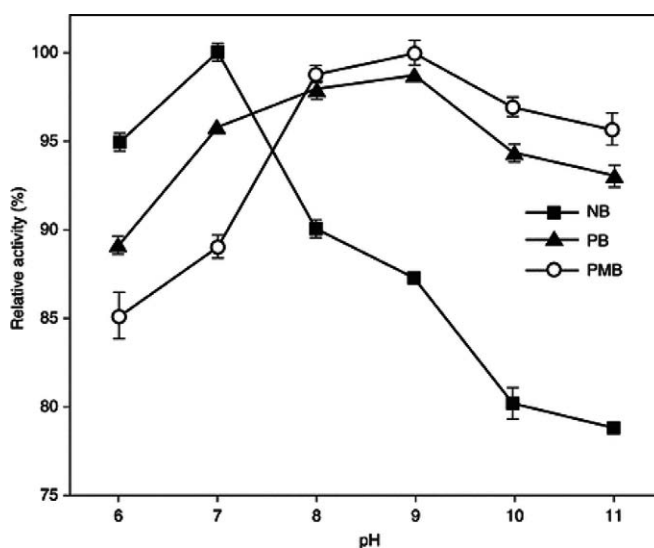

Figure 4. Effect of pH on native bromelain (NB) and on two types of chemically modified bromelain (PB and PMB) activities (40).

creas. For analytical and industrial applications, pancreatin is of porcine or bovine origin. Pancreatin is widely used because of its broad substrate specificity and its ability to hydrolyze proteins, fats, and polysaccharides (41). Its typical applications include: treatment of conditions in which pancreatic secretions are deficient, and use in detergents, and contact lens cleaning solutions (42).

Pancreatin is not stable under conditions of high humidity and temperature. The enzymatic activity reaches a maximum in neutral to weakly alkaline solutions and decreases quickly in acidic or strong alkaline solutions. Pancreatin has a good proteolytic activity in the pH range of 6–8, depending on whether its source is bovine or porcine (see Figure 5) (41).

For use in dissolution testing, the component of interest is the protease activity. The substrate most often used in the determination of pancreatin protease activity is casein (43).

DISSOLUTION TESTING OF CROSS-LINKED GELATIN CAPSULES USING ENZYMES
Objective
The dissolution testing of non-cross-linked gelatin capsules using media with and without enzymes were compared to evaluate the impact of the use of enzymes in the dissolution results. The typical dissolution results were also compared using pepsin and pancreatin. Dissolution testing of cross-linked gelatin capsules was carried out using the enzymes papain and bromelain to determine the suitable amounts of each enzyme capable of digesting the cross-linked gelatin.

Materials and Methods
Pepsin (lot 00511010, 1:10, 400–450 units/mg solid), pancreatin (lot 52320708, protease activity of 203 USP

Figure 5. Proteolytic activity of pancreatin according to the pH of the medium (1 – pig, 2 – bovine) (41).

units/mg), papain (Lot 70310665-B, 66,900 USP units/mg) and bromelain (Lot 55910802-B, 2450 gelatin digesting units (GDU)/ g) were obtained from American Laboratories (Omaha, NE). Aqueous solution of formaldehyde (37%) was purchased from J.T. Baker (Phillipsburg, NJ). Acetaminophen, USP grade was purchased from Sigma-Aldrich (St. Louis, MO). Glacial acetic acid was purchased from J.T. Baker (Phillipsburg, NJ). Buffer salts were reagent grade and purchased from J.T. Baker. Tylenol (Acetaminophen) 500 mg capsules were from McNeil Consumer Healthcare. Gelatin capsules were size 0, clear, Capsugel Lot 70836321.

Preparation of Cross-linked Capsules
Capsules were cross-linked by placing six empty capsules in a round desiccator 15 cm in diameter. In the bottom of the desiccator, 100 mL of a 37% formaldehyde solution was placed and the desiccator was closed to allow interaction between the gelatin capsules and formaldehyde vapors. After 30 min, the capsules were removed, filled with 380 ± 10 mg of pure unformulated acetaminophen, and immediately subjected to dissolution testing. Previous experience had indicated that this procedure would produce a moderate to high degree of cross-linking (44, 45). Alternatively, the pre-filled capsules can be treated by completely vaporize small amounts (i.e., around 5 μL) of 37% formaldehyde by applying vacuum for about 15 seconds to the desiccator, which contains the gelatin capsules samples. The hard gelatin capsules were exposed to formaldehyde for about two hours (44, 45). The dissolution test was carried out just after the forced cross-linking procedure. Other forced cross-linking procedures can be used as long as consistent results are obtained.

The dissolution testing are performed using USP apparatus 2 at 50 rpm with 900 mL of dissolution medium. Helix wire sinkers were used to prevent capsules from floating on the surface of the medium. Dissolution samples were (i) collected at specified time points and analyzed by HPLC, or (ii) the data were generated by a Fiber Optic–UV (FO–UV) system. When FO-UV system was used, the excipient and enzyme background were checked and corrected. There was no significant background interference for acetaminophen detection.

The Impact of Enzymes in Dissolution Results
For each dissolution method, the first step was to verify if there is any impact to the dissolution results by adding the enzymes. The dissolution profiles were obtained using USP apparatus 2 at 50 rpm with 900 mL of dissolution medium. Helix wire sinkers were used to prevent capsules from floating on the surface of the medium. The impact of using pepsin and pancreatin in the dissolution was evaluated using acetaminophen capsules according to the conditions in <711>, and the results showed there is no difference of the dissolution profiles when pepsin (Figure 6) and pancreatin (Figure 7) were added to the dissolution media.
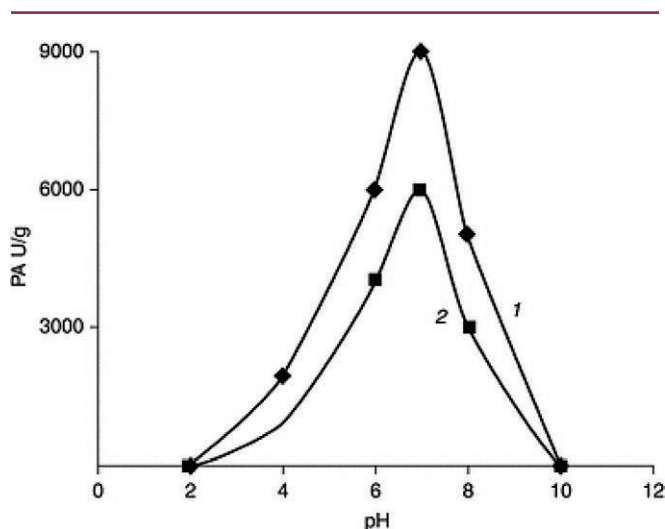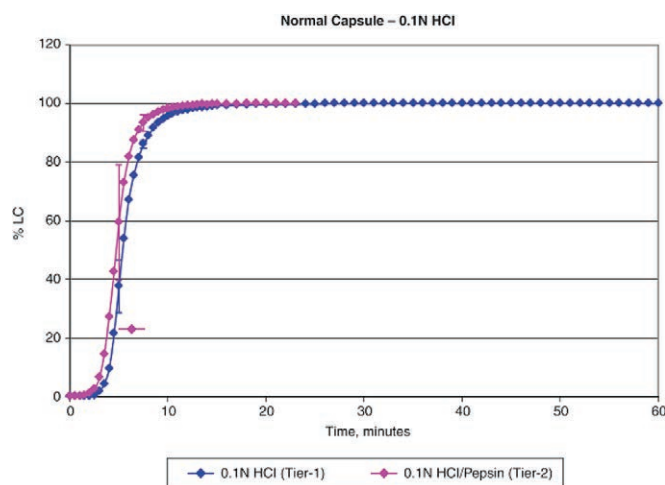
Figure 6. Dissolution profiles of non-cross-linked acetaminophen capsules (normal capsules) using 0.1 N hydrochloric acid as medium, without pepsin (Tier 1) (blue line), with pepsin (Tier 2)(pink line).
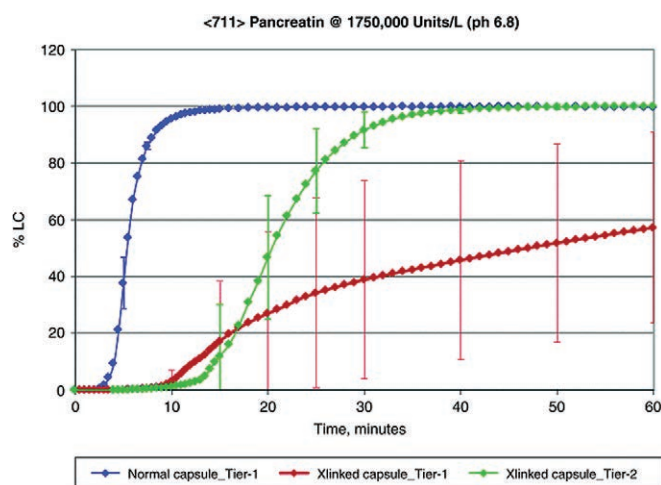

Figure 8. Dissolution profiles of acetaminophen capsules using 0.1N hydrochloric acid as dissolution medium. The blue profile represents a typical dissolution result of non-crosslinked (normal) capsules in the absence of enzyme (Tier-1). Cross-linked gelatin capsules fail the test in the absence of enzyme (Tier-1) (red profile). Adding pepsin per <711> to 0.1 N hydrochloric acid, the cross-linked capsules will pass Tier-2 testing (green profile).
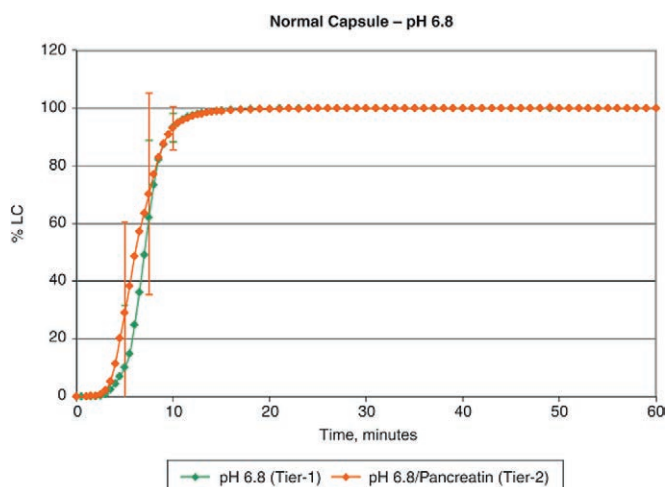

Figure 7. Dissolution profiles of non-cross-linked acetaminophen capsules (normal capsules) using pH 6.8 phosphate buffer as dissolution medium: without pancreatin (Tier-1) (green) and with pancreatin (Tier-2) (orange).
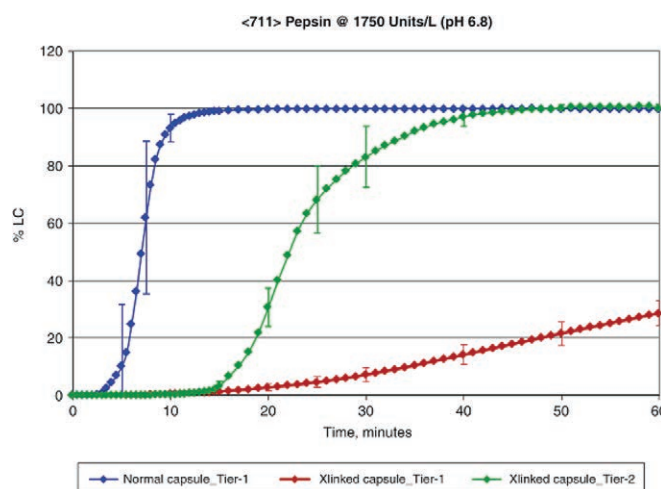

Figure 9. Dissolution profiles of acetaminophen capsules using pH 6.8 phosphate buffer as dissolution medium. The blue profile represents a typical dissolution result of non-cross-linked (normal) capsules without the enzyme (Tier-1). Cross-linked gelatin capsules fail the test in the absence of enzyme (Tier-1) (red profile). Adding pancreatin per <711> to pH 6.8 dissolution media, the same cross-linked capsules will pass Tier-2 testing (green profile).

The second step was to evaluate how a cross-linked capsule fails the dissolution, and how much the dissolution profile can be revived by adding enzymes. Figure 8 shows the dissolution results obtained with cross-linked acetaminophen capsules tested using 0.1 N hydrochloric acid as dissolution medium with and without pepsin. Figure 9 shows the same sample tested using pH 6.8 phosphate buffer as dissolution medium with and without pancreatin. It can be observed that the cross-linked gelatin capsules will fail the test in the absence of enzymes using both dissolution media. The failure can be recovered by using pepsin and pancreatin at the levels stated in <711>.

New Enzymes (Papain and Bromelain)—the Digestion Power and Working pH Ranges

Initially dissolution testing was performed with non-cross-linked capsules, cross-linked capsules with no enzymes, and cross-linked capsules with 0.5 g/L of bromelain (about 2450 GDU/L) and 0.1 g/L of papain (about 6,690,000 U/L) added to the dissolution medium. The enzyme levels were arbitrarily chosen to make sure these

enzymes could provide sufficient digestion power to the cross-linked gelatin.

The dissolution profiles were obtained using USP apparatus 2 at 50 rpm with 900 mL of dissolution medium. Helix wire sinkers were used to prevent capsules from floating on the surface of the medium. Dissolution tests were performed at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 6.8. At pH 4.0–5.5 the dissolution media were 0.05 M acetate buffer, and at pH 6.0 and above the dissolution media were 0.05 M phosphate buffer. At each pH four dissolution tests were performed, one test with reference (non-cross-linked) capsules, one test with cross-linked capsules and no enzymes, one test with bromelain at 0.5 g/L, and one test with papain at 0.1 g/L. Five mL samples were pulled from each vessel after 5, 10, 15, 30, 45, 60, and 75 minutes and five mL of fresh dissolution medium was added to each vessel.

This study was intended to demonstrate that bromelain and papain would be effective in digesting cross-linked gelatin capsules across the pH range of 4.0 to 6.8. Figures 10–17 show the dissolution results of non-cross-linked capsules (normal) tested with dissolution medium without enzymes, cross-linked capsules tested with dissolution medium without the enzyme, cross-linked gelatin capsules tested using dissolution medium containing 0.5 g/L of bromelain and cross-linked gelatin capsules tested using dissolution medium containing 0.1 g/L of papain at various pH values.

The results confirmed that both bromelain and papain are suitable to be used for dissolution testing of cross-linked gelatin capsules in the pH range from 4.0 to 6.8.

Evaluation of the Amounts of Enzyme to Be Used in the Dissolution Testing of Cross-Linked Gelatin Capsules

Using the results from the studies described above, additional studies were carried out to explore the appro-

Figure 11. Dissolution profiles of cross-linked gelatin capsules using the enzymes bromelain in pH 4.0 acetate buffer, and papain in pH 4.0 acetate buffer. The solid blue line is the dissolution profile on non-cross-linked gelatin capsules (reference capsules) containing acetaminophen in pH 4.0 acetate buffer.

Figure 12. Dissolution profiles of cross-linked gelatin capsules using the enzymes bromelain in pH 4.5 acetate buffer, and papain in pH 4.5 acetate buffer. The solid blue line is the dissolution profile on non-cross-linked gelatin capsules (reference capsules) containing acetaminophen in pH 4.5 acetate buffer.
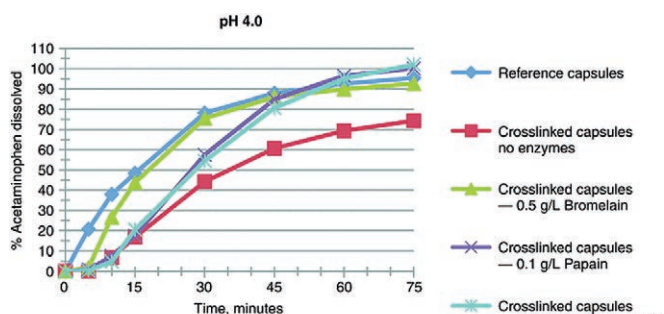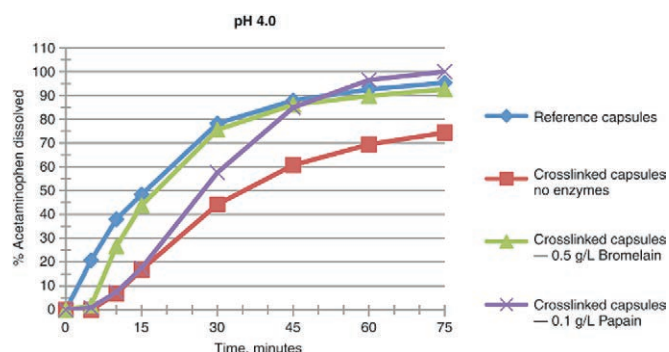

Figure 10. Dissolution profiles of cross-linked gelatin capsules using the enzymes pepsin in 0.1 N hydrochloric acid, bromelain in pH 4.0 acetate buffer, and papain in pH 4.0 acetate buffer. The solid blue line is the dissolution profile on non-cross-linked gelatin capsules (reference capsules) containing acetaminophen in pH 4.0 acetate buffer.
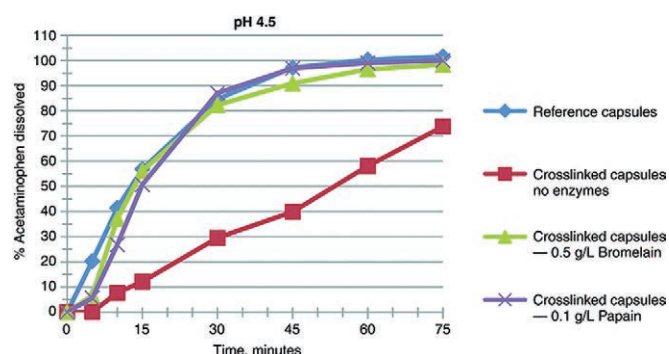

Figure 13. Dissolution profiles of cross-linked gelatin capsules using the enzymes bromelain in pH 5.0 acetate buffer, and papain in pH 5.0 acetate buffer. The solid blue line is the dissolution profile on non-cross-linked gelatin capsules (reference capsules) containing acetaminophen in pH 5.0 acetate buffer.

Figure 14. Dissolution profiles of cross-linked gelatin capsules using the enzymes bromelain in pH 5.5 acetate buffer, and papain in pH 5.5 acetate buffer. The solid blue line is the dissolution profile on non-cross-linked gelatin capsules (reference capsules) containing acetaminophen in pH 5.5 acetate buffer.
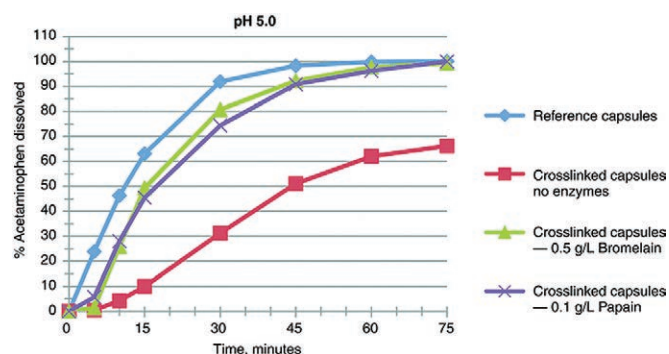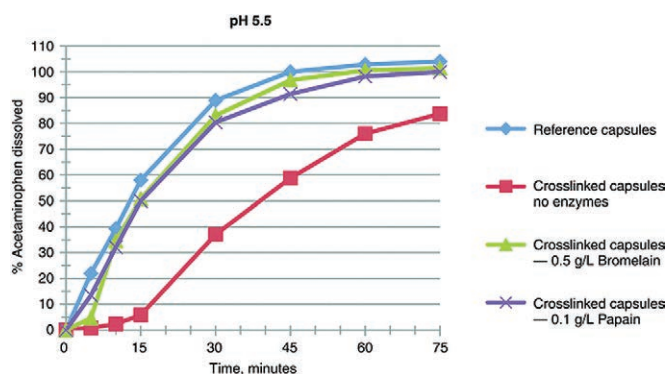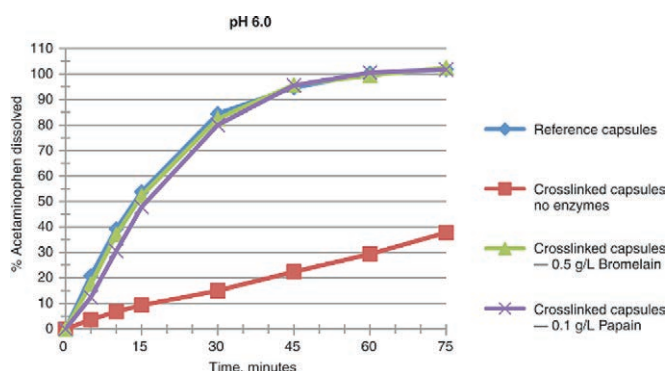

Figure 15. Dissolution profiles of cross-linked gelatin capsules using the enzymes bromelain in pH 6.0 phosphate buffer, and papain in pH 6.0 phosphate buffer. The solid blue line is the dissolution profile on non-cross-linked gelatin capsules (reference capsules) containing acetaminophen in pH 6.0 phosphate buffer.

Figure 16. Dissolution profiles of cross-linked gelatin capsules using the enzymes bromelain in pH 6.5 phosphate buffer, and papain in pH 6.5 phosphate buffer. The solid blue line is the dissolution profile on non-cross-linked gelatin capsules (reference capsules) containing acetaminophen in pH 6.5 phosphate buffer.

priate enzyme levels to be used for each selected enzyme (pepsin, papain, bromelain, and pancreatin). It was found that the gelatin dissolution rate (Figure 18) is faster in pH around 1.2 (0.1 N hydrochloric acid) than in pH 4.5 (acetate buffer) and pH 6.8 (phosphate buffer). This indicates that the capsule opening is faster and in favor of drug release from the capsules in 0.1 N hydrochloric acid.

The cross-linked capsules samples were prepared by treating the hard gelatin capsules with formaldehyde (*44, 45*). The hard gelatin capsules were exposed for two hours and the dissolution test performed immediately after the treatment to control the consistency of gelatin cross-linking level.

The dissolution profiles obtained with cross-linked and non-cross-linked capsules were compared. The cross-linked capsules failed the dissolution test at all three pH

Figure 17. Dissolution profiles of cross-linked gelatin capsules using the enzymes bromelain in pH 6.8 phosphate buffer, and papain in pH 6.8 phosphate buffer. The solid blue line is the dissolution profile on non-cross-linked gelatin capsules (reference capsules) containing acetaminophen in pH 6.8 phosphate buffer.
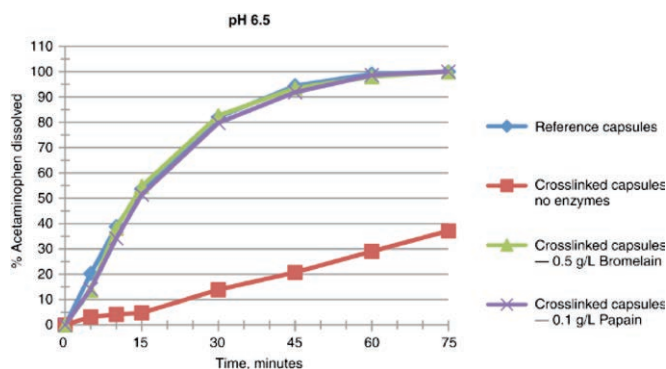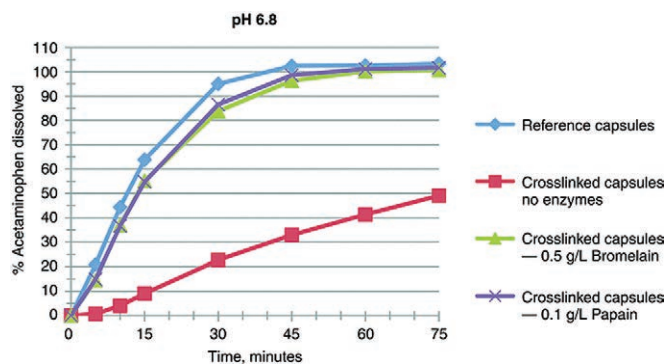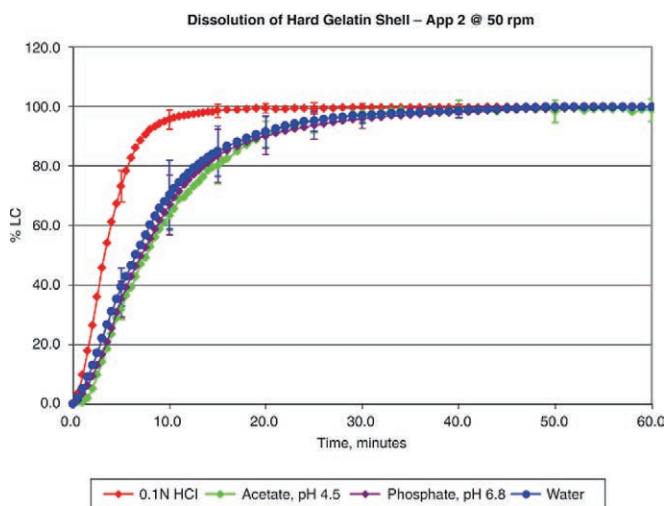

Figure 18. Dissolution profiles of hard gelatin capsule shells in various dissolution media.

conditions. The cross-linked capsules passed the test when pepsin was used with 0.1 N hydrochloric acid (see Figure 8).

Using all the background information available, it was decided to use as a reference the dissolution profile obtained with 0.1 N hydrochloric acid with pepsin as a reference to evaluate the performance of the other dissolution conditions. Different amounts of bromelain or papain were added to 50 mM pH 4.5 sodium acetate buffer, and different amounts of pancreatin were added to 50 mM pH 6.8 sodium phosphate buffer. The goal was to identify the proper amount of each enzyme to achieve similar effectiveness in digesting cross-linked gelatin as was obtained using pepsin in 0.1 N hydrochloric acid containing pepsin. The following conditions show similar results in the ability of the used level of enzyme to digest cross-linked gelatin hard capsules:

- pepsin in pH below 4 : 750,000 units/L (as the current version of <711>)
- pancreatin in pH equal or above 6.8 : 2000 units/L
- bromelain in the pH range 4–6.8 : 30 GDU/L
- papain in the pH range 4–6.8 : 550,000 units/L

Figure 19 shows the dissolution profiles obtained using these enzymes in the amounts mentioned above.

Capsule Opening and Drug Release Behaviors

The drug release process from capsule products may go through two major steps, i.e., (i) capsule shell opening, and (ii) drug release from the content/fill. From the acetaminophen capsule experiments, the dissolution data was analyzed taking into consideration the time necessary to release a certain amount of drug, since the data were continuously collected by a FO–UV system. The drug released at >5% of label claim (LC) (i) was used to reflect a stage that the capsule shell has significantly opened, and (ii) at >85% of LC to show the full drug release stage. The time (in minutes) to reach each stage from each trial was identified from the detailed profiles obtained by the FO-UV system. Figure 20 shows that non-cross-linked capsules (good capsules) opened and released at least 5% of LC within 4 to 6 minutes in three different dissolution media. When cross-linked capsules were evaluated, the initial release of 5% was delayed by as much as 20 minutes. The cross-linked gelatin was not completely dissolved during the testing time period, and the drug release was achieved through some small holes (openings) of the capsule shell, which limited the content from spreading out. Therefore, the drug release level never reaches 85% LC at 60 minutes (i.e., the end of the dissolution run) when the dissolution media did not contain enzymes. This behavior caused the dissolution testing to fail the specification of Q = 80% at 30 minutes.

The same capsules were tested with dissolution media in the absence of enzymes, and the results are shown in Figure 20. For good capsules, there is no significant difference for the drug release rate from the Tier-1 results. The drug release processes compared for the four different pH conditions are very similar. It showed the capsule opening time changed from 3–5 minutes to 11–15 minutes; the gelatin capsule continued to dissolve and the drug release reached full release within no more than 12 minutes after the capsule opened. The cross-linked acetaminophen capsule samples can pass the Q = 80% at 30 minutes by Tier-2 testing under all these three conditions.

The same capsules were tested using dissolution media containing enzymes (Tier-2), and the results are shown in Figure 21. For good capsules, there is no significant difference for the drug release rate from the Tier-1 results. The drug release processes compared for the four different enzyme/pH conditions are very similar. It showed the cap-
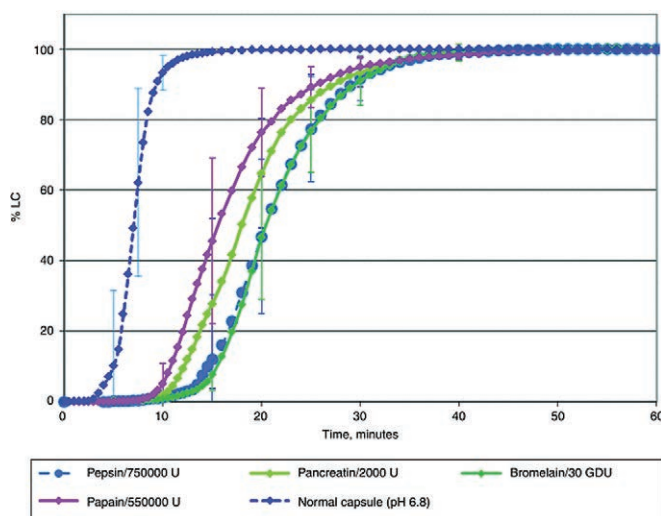

Figure 19. Dissolution profiles of cross-linked gelatin capsules using the enzymes pepsin in 0.1 N hydrochloric acid, pancreatin in pH 6.8 phosphate buffer, bromelain in pH 4.5 acetate buffer, and papain in pH 4.5 acetate buffer. The blue dash line is the dissolution profile of non-cross-linked gelatin capsules containing acetaminophen in pH 6.8 phosphate buffer.
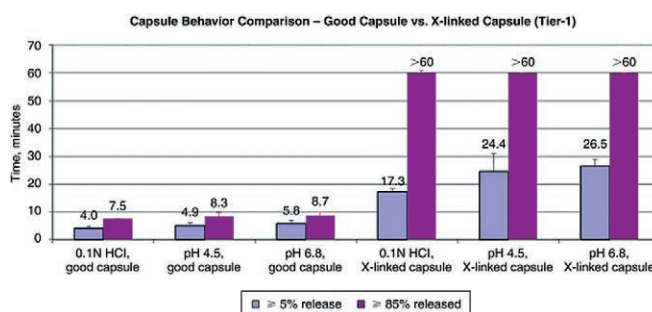

Figure 20. Drug release and capsule behavior comparison for non-cross-linked capsule versus cross-linked capsule in the absence of enzymes (Tier-1).
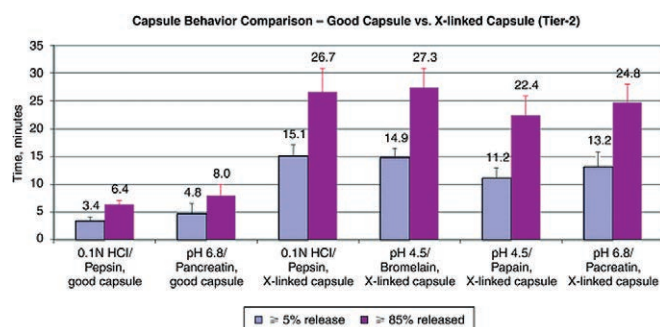
Figure 21. Drug release and capsule behavior comparison for non-cross-linked capsules (good capsules) vs. cross-linked capsules using dissolution media containing enzymes (Tier-2).

sule opening time changed from 3–5 minutes to 11–15 minutes; the gelatin capsule continued to dissolve and the drug release reached full release within no more than 12 minutes after the capsule opened. The cross-linked acetaminophen capsule samples can meet the acceptance criteria of Q = 80% at 30 minutes by Tier-2 testing under all these four conditions.

The time to reach 5% of LC can be used for the decision of the pre-treatment time when enzymes are added to the dissolution medium (Tier-2 methods). The time gap between 5% of LC and 85% of LC on the formulation, which needs to be carefully evaluated during method development.

INTERACTION ENZYMES—SURFACTANTS

Surfactants may inhibit enzymatic activity because of denaturation of the enzyme or by competition for the substrate, or surfactants can enhance enzymatic activity. The type of interaction depends on the type of surfactant, the substrate being used with the enzyme, the solvent, the pH of the medium, and the characteristics of the enzyme. A comprehensive review of the interaction of enzymes with surfactants was published by Otzen (46). With very few exceptions, non-ionic surfactants do not denature proteins, whereas ionic surfactants do so at very low concentrations that are often well below their critical micelle concentration (CMC). The critical issue is not the concentration of surfactant present but the ratio between protein and surfactant, because this will determine how much surfactant is available per protein molecule. An additional layer of complexity is introduced if the surfactant changes ionization over a pH range that is relevant for the protein. In this case, small changes in pH can have profound effects on protein-surfactant interactions.

Given the multiple ways in which surfactants can bind to proteins, it is expected that they can have other effects on proteins besides denaturing them or stabilizing them as ligands. Many proteins are stabilized by the binding to ionic surfactants. Some proteins are simply resistant

to denaturation by a specific surfactant such as sodium lauryl sulfate (SLS). Examples of non-SLS-binding proteins include papain and pepsin. (46). SLS, dodecyl trimethyl-ammonium chloride and Peregal O (non-ionic surfactant) in various concentrations were shown to increase the enzymatic activity of pancreatin when the substrate was gelatin (47). The effect of SLS and Tween 80 on the dissolution of cross-linked gelatin capsules using pepsin and pancreatin was evaluated. SLS significantly hampered the dissolution due to the formation of a less soluble precipitate of gelatin.

Another study found similar results for SLS (48). SLS significantly reduced the dissolution of gelatin capsules below pH 5. Visually, the gelatin shells were transformed into a less-soluble precipitate under these conditions. The precipitate was found to have a higher sulfur content than the gelatin control sample, indicating that SLS is part of the precipitate. Tween 80 showed far better results in dissolution media at neutral or low pH (49).

The inhibitory effect of dioctyl sodium sulfosuccinate on pepsin activity was investigated over the pH range of 1.5–3.0. The inhibitory effect was studied using a natural substrate, hemoglobin, and a synthetic substrate, N-acetyl-L-phenylalanyl-L-diiodotyrosine. A substrate-inhibitor interaction was the major mechanism of inhibition with hemoglobin. With the synthetic substrate, the inhibition was due to a competition between the substrate and the inhibitor molecules for the enzyme (50). Papain is denatured by SLS (51) but its activity can be enhanced with moderate amounts of Tween 80 (52). Analysis of kinetic data showed that SLS acts as a partial non-competitive inhibitor for bromelain, and demonstrated that bromelain is resistant to SLS binding and denaturation (53).

The influence of some surfactants on the pepsin activity was evaluated at the USP Biologics and Biotechnology Laboratory. The FCC (18) procedure for determination of pepsin activity employs 30 mM hydrochloric acid, pH 1.6, as diluent. This diluent was replaced by the following solutions, commonly used as dissolution medium, hydrochloric acid adjusted to pH 1.2, 1.6, and 2.2; 0.1 M phosphate buffer, pH 2.5; pH 3.0 and pH 4.0 citrate buffer; 0.2 M acetic acid buffer pH 4.0. The results showed that buffers with pH values of 2.5 and below did not inhibit the protease activity of pepsin. Citrate buffer at pH 3.0 reduced the activity by 31%. Acetic buffer and citrate buffer, both at pH 4.0, almost completely inhibited the pepsin activity. Surfactants were added to the diluent used in the FCC procedure (30 nM hydrochloric acid, pH 1.6). Tween 80, Tween 20, and SDS were tested at the concentrations of 0.1% and 0.5%. SDS at 0.1% did not inhibit the pepsin activity but 0.5% SDS inhibited the activity by 75%. Tween 20 and Tween 80 did not inhibit pepsin activity at the concentrations of 0.1% or 0.5%; rather both surfactants seemed to enhance the activity of pepsin by as much as 80%.

Because the enzyme-surfactant interaction is influenced by so many variables and the outcome cannot be easily predicted, if the dissolution medium used in the dissolution of gelatin capsules or gelatin coated tablets contains surfactants, it is recommended to carefully verify the impact from surfactant in the enzyme digestion power needed in the dissolution test. If the loss of enzyme activity is clearly observed, a pre-treatment can be applied.

The dissolution procedure that includes a pre-treatment step uses two dissolution media: Medium A is the dissolution medium for the pre-treatment of the cross-linked gelatin capsule or gelatin-coated tablet. It is the original medium in the dissolution procedure with the appropriate enzyme but without the surfactant or solubility enhancer. Medium B is the original medium in the dissolution procedure containing the surfactant or solubility enhancer at a concentration sufficient to produce the concentration in the original dissolution medium when added to Medium A. Other than adding the enzyme, the original composition of the dissolution medium cannot be modified for the pre-treatment step. In some publications the original procedure is called Tier 1 and the procedure with the addition of the enzyme is called Tier 2.

Pre-treatment Step Method Development

The pH of the dissolution medium determines the appropriate enzyme to be used according to <711>. The amount of the enzyme to be added to the dissolution medium should be experimentally to obtain the required protease activity. The stability of the enzyme in the pre-treatment dissolution medium needs to be evaluated to optimize the method performance.

For the pre-treatment procedure, the volume of Medium A is smaller than the volume stated in the dissolution procedure. This volume is defined on a case-by-case basis. After the pre-treatment period, a volume of Medium B is added to the vessel to reach the specified dissolution medium volume and the final surfactant or solubility enhancer concentration as stated in the original procedure. Medium B should be pre-warmed to 37.0 ± 0.5 °C before it is added to Medium A. The addition should be as gentle as possible to minimize the physical disturbance to the dissolution medium flow. The agitation should be maintained during the pre-treatment step and it should be the same as stated in the original method or monograph.

Typically, the pre-treatment time period is not more than 15 minutes. However, the required pre-treatment time should be evaluated on a case-by-case basis and should be justified. This time is part of the total time of the dissolution procedure.

Some examples of this pre-treatment step can be found in the "FDA-Recommended Dissolution Methods" database available at http://www.accessdata.fda.gov/scripts/cder/dissolution/index.cfm (Figure 22).

Drug name	Dosage form	USP apparatus	Speed (RPMs)	Medium	Volume (mL)	Recommended sampling times (minutes)	Date updated
Ziprasidone HCl	Capsule	II (Paddle)	75	Tier I: 0.05 M Na phosphate buffer, pH 7.5 + 2% SDS (w/w) Tier II: 0.05 M Na phosphate buffer, pH 7.5 (700ml) + 1% pancreatin, after 15 min. incubation, add 200 mL of phosphate buffer containing 9% SDS	900	10, 20, 30, 45 and 60	03/04/2006

Figure 22. Example of dissolution procedure with the pre-treatment step from the FDA-recommended Dissolution Methods database (http://www.accessdata.fda.gov/scripts/cder/dissolution/index.cfm).

By using the procedure described in Figure 22 as an example, the dissolution medium is 900 mL of 0.05 M sodium phosphate buffer, pH 7.5, containing 2% (w/w) sodium dodecyl sulfate (SDS, synonymous with SLS). In the presence of cross-linking, the samples undergo a 15 min pre-treatment where the medium is 700 mL of 0.05 M sodium phosphate buffer, pH 7.5, containing 1% of pancreatin. After 15 min, 200 mL of 0.05 M sodium phosphate buffer, pH 7.5, containing 9% of SDS is added to the vessel and the test is continued until the final time point.

The stepwise addition of enzyme and surfactant enables both agents to take effect individually and sequentially in the dissolution medium. The enzyme digests the cross-linked gelatin capsule shell at the beginning to ensure the opening of the capsul shell, whereas the addition of surfactant or solubility enhancer afterwards increases drug solubility and/or wettability. The pre-treatment can be used even when the surfactant is a component of the formulation. Once the cross-linked capsule shell ruptures and dissolution starts, the surfactant inside the formulation will work as expected (*14*). Because most of the dissolution parameters remain unchanged, the development of the pre-treatment step should focus on the selection of the enzyme, adjustment of the volumes of Medium A and Medium B, definition of the concentration of surfactant or solubility enhancer in Medium B, and determination of the time for Medium A pre-treatment.

Pre-treatment Step Method Validation

Specificity, accuracy, precision, linearity, and range are the primary parameters in the validation of any typical dissolution method. Some additional considerations may apply to a pre-treatment procedure (*54*). For specificity, non-interference of the enzyme should be confirmed using the pre-treatment procedure. Accuracy, precision, linearity, and range should not be compromised due to the addition of the enzyme. The laboratory should use the proposed pre-treatment procedure to verify the repeatability of the procedure on the test article at appropriate time points

The dissolution test using enzymes, with or without the pre-treatment, 7 may be implemented during the course of stability studies, provided that the method has been properly validated. The pre-treatment step is intended to assess the degree of the release from the

test article at the original specification time points. The dissolution profile from the pre-treatment method may not be comparable to that generated using the original dissolution method.

OTHER RELATED REVISIONS

During the development of the revision being made to Dissolution <711> and Disintegration and Dissolution of Dietary Supplements <2040> or as a consequence of this revision, other monographs and general chapters will be reviewed:

- <1094> *Capsules—Dissolution Testing and Related Quality Attributes*
- <1092> *The Dissolution Procedure—Development and Validation*
- *Pepsin*, as reagent
- *Bromelain*, as reagent
- New monograph for *Hard Gelatin Capsule Shell*
- *Gelatin* monograph

We strongly recommend that readers monitor *Pharmacopeial Forum* for future revisions to these monographs and chapters. Readers should also send any comments, questions, suggestions, or data to Margareth Marques at MRM@usp.org.

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