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Crosslinking of gelatin capsules and its relevance to their *in vitro-in vivo* performance

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Abstract

The present review deals with the chemistry of gelatin crosslinking under conditions that are relevant to pharmaceutical situations. Mechanistic rationalizations are offered to explain gelatin crosslinking under "stress" conditions. These include elevated temperature and high humidity conditions. In addition, the chemical interactions between gelatin and aldehydes, such as formaldehyde and other formulation excipients, are discussed.

The literature on the *in vitro* and *in vivo* dissolution and bioavailability of a drug from stressed gelatin capsules and gelatin-coated tablets is reviewed. Crosslinking phenomena, occurring in stressed hard gelatin capsules and gelatin-coated tablets, could cause considerable changes in the *in vitro* dissolution profiles of drugs. However, in a few cases, the bioavailability of the drug from the stressed capsules is not significantly altered when compared to that obtained from freshly packed capsules. It is concluded that, as with other drug-delivery systems, careful attention should be paid to the purity and chemical reactivity of all excipients that are to be encapsulated in a gelatin shell. It is suggested that *in vitro* dissolution tests of hard gelatin-containing dosage forms be conducted in two stages, one in a dissolution medium without enzymes and secondly in dissolution media containing enzymes (pepsin at pH 1.2, and pancreatin at pH 7.2, representing gastric and intestinal media, respectively) prior to *in vivo* evaluation. Such *in vitro* tests may constitute a better indication of the *in vivo* behavior of gelatin-encapsulated formulations. Furthermore, testing for contamination with formaldehyde as well as low molecular weight aldehydes should be a standard part of excipient evaluation procedures.

Introduction

Gelatin is a mixture of water-soluble proteins derived primarily from collagen. It is obtained by boiling skin, tendons, ligaments, bones, and other similar products in water. Gelatin is extensively used in solid dosage forms: hard and soft gelatin capsules, and gelatin-coated tablets. It is a favorable material for use as the outer layer of drug formulations because of its glossy appearance, ability to hold dye color, neutral taste, and processing convenience. Gelatin has also been used as a stabilizer, thickener, and texturizer in foodstuffs.

Due to the apparent dissolution problems in *in vitro* testing of hard and soft capsules, questions have been raised about the potential of crosslinking effects in gelatin (1). The current literature tends to indicate that these effects primarily impact the *in vitro* testing methodology rather than the *in vivo* bioavailability of drugs formulated in hard gelatin capsules. Nevertheless, this chemical reactivity of gelatin must be taken into account when designing the final drug formulation.

In general, the amino acid content of gelatin is glycine 25.5 %; proline 18 %; hydroxyproline 14.1 %; glutamic acid 11.4 %; alanine 8.5 %; arginine 8.5 %; aspartic acid 6.6 %; lysine 4.1 %; leucine 3.2 %; valine 2.5 %; phenylalanine 2.2 %; threonine 1.9 %; isoleucine 1.4 %; methionine 1.0 %; histidine 0.8 %; tyrosine 0.5 %; serine 0.4 %; cystine and cysteine 0.1 % (2).

Gelatin may be produced by lime hydrolysis of bones and animal skins (Type B, isoelectric point = 4.7 to 5.3) or by acid hydrolysis of animal skins (Type A, isoelectric point = 6.0 to 8.0) (1). Hard gelatin

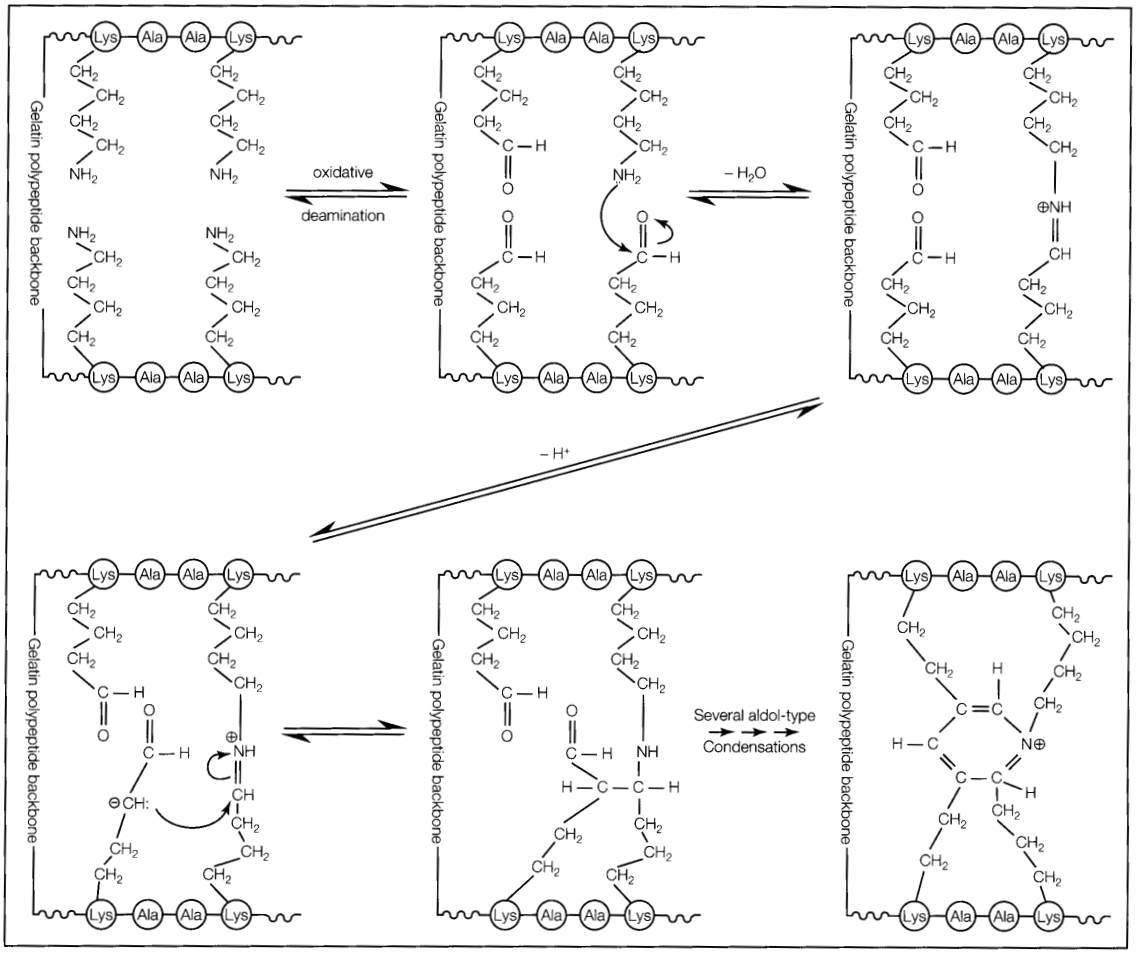
capsules are usually prepared from a mixture of Type A and B gelatins.

The reactivity of the gelatin molecule appears to arise from the trifunctional amino acids it contains, specifically, lysine. In Type B gelatin, the lysine-derived ε-amino function content was found to be 33.0 moles per gelatin molecule of 1,000 amino acid residues (3). Carboxyl groups are far more plentiful than amino groups in gelatin but appear to be less reactive in reactions that involve crosslinking (1). The amino groups arising from histidine residues are generally thought not to play as an important role in gelatin crosslinking as the ε-amino groups of lysine (1). The highly basic guanidino group of arginine is protonated at neutral pH and consequently is thought to be unreactive unless a very high pH is employed (1). More recently, however, the participation of arginine in the hardening of gelatin in the presence of formaldehyde has been shown to be eminent (4-6).

Chemistry of gelatin crosslinking

The crosslinking of the gelatin molecule is a well-known phenomenon that may occur by one or several chemical reactions (7). One possible reaction may involve the formation of desmosine-type crosslinking, known to occur in elastin fiber (8). Lysine residues which are proximal to each other are oxidatively deaminated to yield terminal aldehyde groups. One of the aldehyde groups could then be attacked by a free ε-amino group of a neighboring lysine to yield an imine which subsequently undergoes a series of aldol-type condensation reactions to produce a crosslinked product containing pyridinium ring(s) (8), (Scheme 1).

Another possible gelatin crosslinking event involves the reaction of a lysyl ε-amino group with an aldehyde (Scheme 2). (Trace quantities of formaldehyde may be present in corn starch (9-12), which is an excipient in many drug formulations). The initial



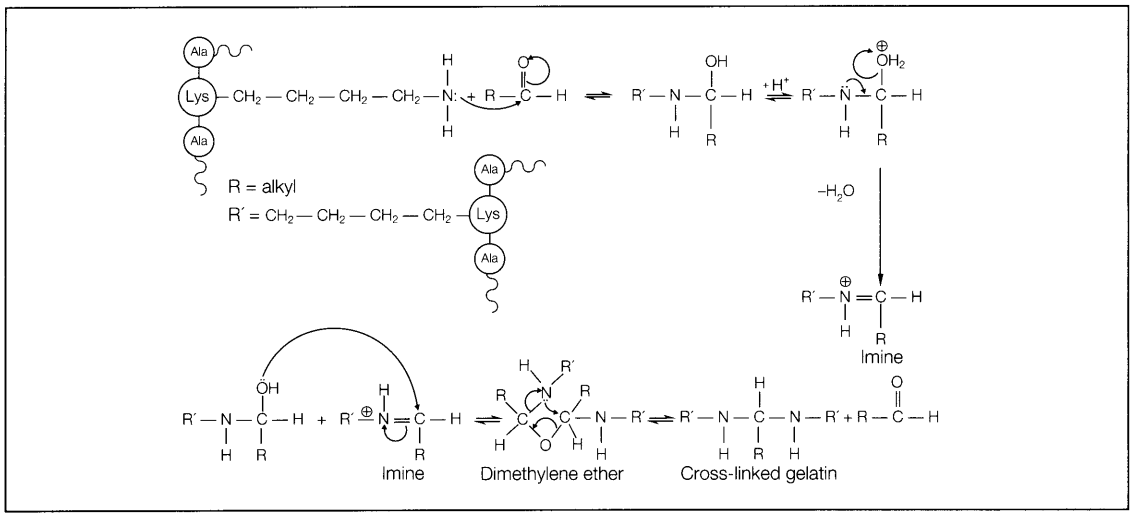
Scheme 1.

product, a hydroxymethylamino group (Scheme 2), eliminates water to give a cationic imine. The imine can react with another hydroxymethylamino lysine residue to yield a dimethylene ether (1). This ether may then rearrange to form a methylene link between two ε-amino groups of lysine.

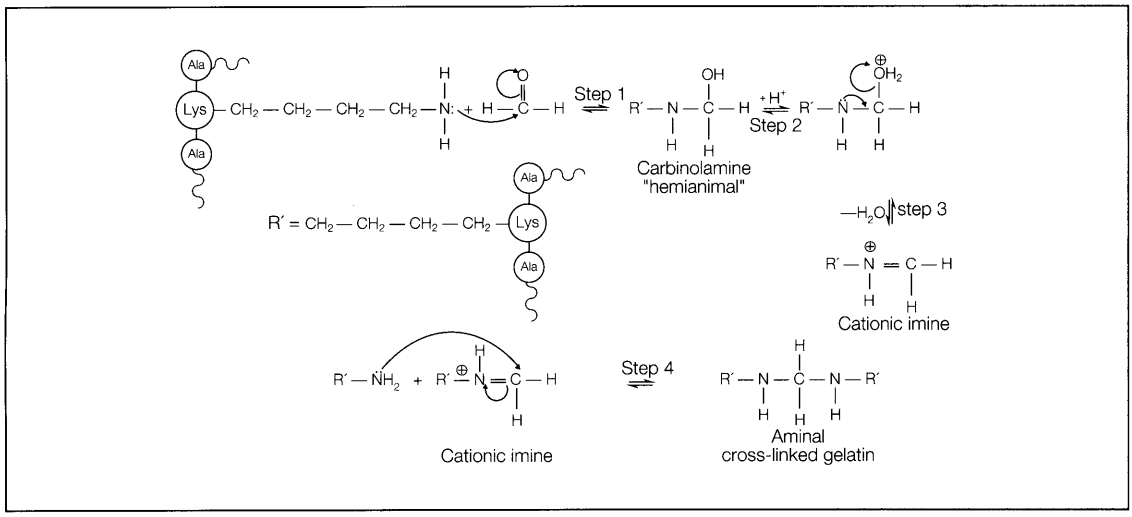
A third example of gelatin crosslinking is the formation of an imine arising from the reaction the ε-amino group of lysine and an aldehyde, with subsequent formation of an aminal, the amine form of an acetal (Scheme 3). Step 1 of Scheme 3 involves the formation of a carbinolamine, which is rate limiting in acid pH, while step 3 involves the formation of the imine with concurrent water loss (13, 14). The latter step (step 2) is rate limiting in base (13, 14).

The final step (step 4) requires the attack of a free amine on the cationic imine to give the aminal (Scheme 3). The optimal pH of formation of the aminal would inevitably be close to 7 because each step (step 1 or step 2) becomes rate limiting on either side of neutral pH. Aminal formation, like that of an acetal, is reversible at low pH. The acidic milieu combined with the presence of enzymes in the stomach explains why crosslinking in gelatin capsules may exert little influence on the *in vivo* dissolution rate of a drug.

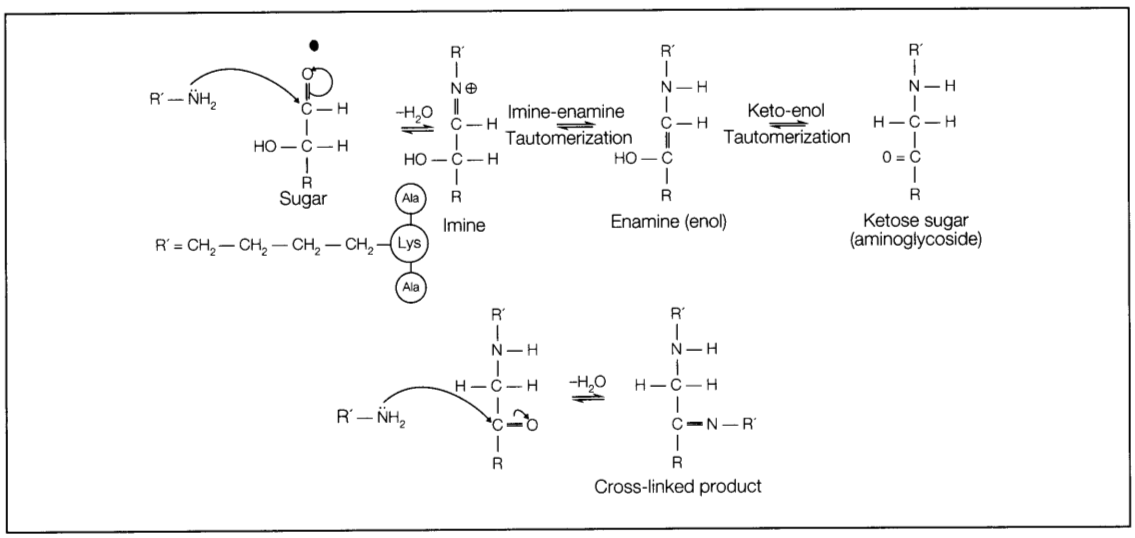
Glucose or other aldose sugars are often included in drug formulations and may provide yet another possibility for gelatin crosslinking. The aldehyde functional group of these saccharides may react with a free ε-amino group to give an imine intermediate,



Scheme 2



Scheme 3



Scheme 4

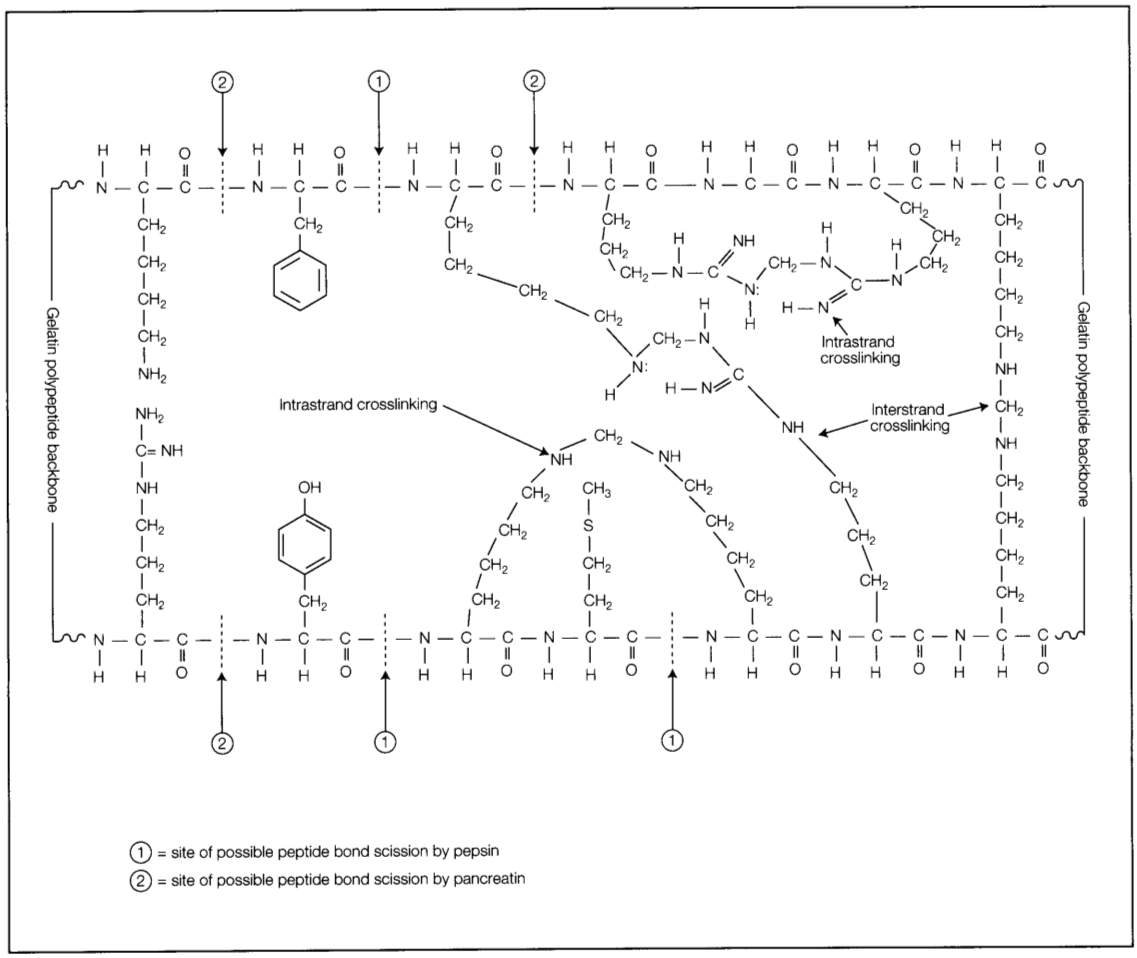


Figure 1

which, through an Amadori rearrangement (15), produces a ketose sugar (Scheme 4). The formed aminoglycoside (ketose sugar) is then free to react with another amine through its carbonyl functionality to form the crosslinked gelatin (9, 15).

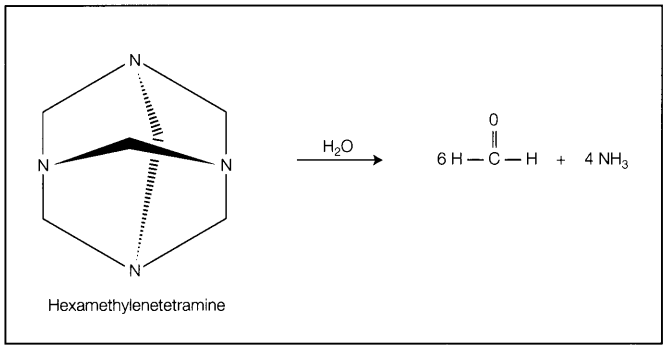
Types of gelatin crosslinking

In general, crosslinking within the gelatin polypeptide may occur in one of two ways. Bridging may take place within the same polypeptide strand (intrastrand, intramolecular crosslinking), or amino acid residues from two neighboring peptide strands may form a bridge (interstrand, intermolecular crosslinking) (Figure 1).

Model reactions of the hardening of gelatin with ^{13}C-labeled formaldehyde have recently been monitored by using ^{13}C NMR spectroscopy in solution and solid state (5). It was found that when a 12 % gelatin solution is treated with 0.5 % ^{13}C-labeled formaldehyde, the latter first formed methylols (carbinolamines) of lysine residues and subsequently of arginine residues. After longer incubation periods with formaldehyde (72 hours) lysine-arginine aminals (crosslinks) are formed (Figure 1). During the drying process of hardened gelatin, in addition to the lysine-arginine crosslink, an arginine-arginine-crosslink appears to form (Figure 1). Humidity strongly influences the rate of this crosslinking process (5).

Agents which catalyze gelatin crosslinking

Gelatin capsules undergo conformational change and crosslinking when stored under high humidity conditions, as demonstrated by gemfibrozil, hydrochlorothiazide, and diphenhydramine hydrochloride capsules (16). The crosslinking process causes formation of a swollen, rubbery, water-insoluble membrane (pellicle) during dissolution testing (17, 19). This water-insoluble gelatin film acts as a barrier, restricting drug release. The mechanism through which humidity probably acts is by indirect catalysis of imine formation, which is the first intermediate in all crosslinking reactions listed in the previous section. For example, corn starch, a drug excipient, may contain traces of the stabilizer hexamethylenetetramine (9, 12), which decomposes under humid conditions to form ammonia and formaldehyde (Scheme 5) (20). The latter may react with lysine residues present in gelatin to form imines, which subsequently can undergo any of the possible crosslinking reactions described above (Schemes 1-4).



Scheme 5

The presence of aldehydes, among other reactants, leads to an increase in the molecular weight of gelatin likeky due to formation of interstrand bridges across its polypeptide backbone (9). This crosslinking reaction has been utilized to prepare gelatin capsules which are resistant to gastric juice (21, 23). Formaldehyde, the main crosslinking agent related to the loss of solubility of gelatin capsules, is believed to react with the ε-amino functions of lysine (1) and the guanidino function of arginine residues present in gelatin (4-6).

In addition to formaldehyde, other aldehydes may present problems to gelatin-encapsulated drug formulations. For example, it has recently been shown that gelatin capsules packaged in bottles containing rayon coilers exhibit decreased *in vitro* dissolution rates after two months at 40 °C and relative humidities of 18 % to 75 % (23). The rayon process, involved in coiler production uses cellulose which contains low levels of hemicellulose (i.e. pentosans), as a starting material. The chemical process for rayon production involves an acid treatment. Under these conditions, hemicellulose produces pentose sugars which, in turn, can form furfural. Subsequently, the aldehydic function of furfural has been shown to react rapidly with gelatin to form a crosslinked insoluble product (23).

Plasticizers and preservatives, fats and polyethylenated compounds such as polyethylene glycols, ethers of polyethyleneglycol and aliphatic alcohols or phenols, polyoxyethylenated glycerides, and nonionic surfactants (polysorbates, esters of unsaturated fatty acids) are frequently used in soft gelatin capsules. These compounds can undergo autoxidation to form aldehydes of higher molecular weights (9, 24). The latter can react with gelatin to form crosslinked products resulting in lower *in vitro* dissolution rates (24). This type of crosslinking is usually absent in hard gelatin capsules since they do not contain the plasticizers or preservatives inherent to soft gelatin capsules.

The influence of ultraviolet light on gelatin capsule crosslinking has been investigated using gemfibrozil, hydrochlorothiazide, and diphenhydramine hydrochloride, along with various dyes present in the capsules (16). It was found that intense UV or visible irradiation promoted changes in the gelatin which resulted in lower *in vitro* dissolution rates. This effect was especially pronounced when the irradiation was combined with conditions of high humidity and gelatin capsules containing FD&C Red No. 3 or FD&C Red No. 40 (16, 25). Many FD&C dyes have been known to interact with gelatin via hydrophobic and hydrogen bonding (1, 26). In addition, Kellaway et al. have demonstrated a correlation between the increase in gelatin molecular weight and the quantity of bound dye (27).

In summary, the presence of aldehydes, dyes, high humidity, and prolonged exposure to light may play a separate or synergistic role in increasing the *in vitro* dissolution times of drug substances from gelatin capsules by altering the disintegration of gelatin capsules. In such cases the capsules are often referred to as being "stressed".

Reversibility of crosslinking

1. Nonenzymatic

The reversibility of the aminal reaction, (Scheme 3, step 4) as well as that of the imine functionality, shows a definite pH dependence (Scheme 3, steps 2 and 3). In general, the aminal decomposition (Scheme 3, step 4) is reversible under basic conditions (13, 14) while step 2 and 3 (imine to carbinolamine) are reversible in acidic media. Carbinolamine decomposition (step 1) occurs under basic conditions (13, 14).

2. Enzymatic

The impeding barrier that is exerted upon drug molecules by a highly crosslinked gelatin capsule wall can be alleviated by the presence of pepsin and/or pancreatin (25, 28, 29). Gastric juice, at pH 1.2, consists mainly of the enzyme pepsin, an acid-dependent endopeptidase which cleaves peptides proximal to hydrophobic and aromatic amino acids (Figure 1) (30).

Likewise, pancreatin, which contains the protease enzyme trypsin, an endopeptidase which operates at neutral pH and preferentially cleaves the carboxyl side of lysine and arginine residues (30), hydrolyzes specific peptide linkages of gelatin, thus liberating the drug which it encapsulates. Figure 1 illustrates the dual effect of pepsin and pancreatin on scission of certain peptide bonds within the gelatin molecule.

Comparison of the *in vitro* dissolution rate of drugs, formulated in gelatin capsules, with their *in vivo* bioavailability

The investigation of the relationship between dissolution rates of drugs and their bioavailabilities in humans is necessary to establish meaningful *in vitro* dissolution tests.

In order to obtain an *in vitro-in vivo* correlation for any product, it is necessary to have products which differ in their *in vivo* performance as well as in their *in vitro* behavior. The *in vivo* performance of the product is invariable and cannot be altered. On the other hand, the *in vitro* parameters are variable, and are dependent on the method, stirring rate, dissolution medium, and other variables and can be adjusted to mimic the *in vivo* performance of the product. The *in vivo* parameters such as AUC (area under the plasma concentration curve), t_{max} (time to reach maximum concentration), C_{max} (maximum concentration), or Ae (cumulative amount of drug excreted unchanged in urine) of the products are obtained from the bio-studies and are commonly utilized in the correlation. The *in vitro* dissolution rates of the products are generally determined using basket or paddle method (USP Apparatus 1 or 2) in a suitable dissolution medium at appropriate degree of agitation, and percent dissolved at a specified time or the time needed to dissolve certain percentage are utilized in the correlation. The key elements are the proper choice of the medium and degree of agitation. In developing an *in vitro* test, it is important not to make the method more complicated than is necessary, and also not to develop a method that is overly discriminatory. In order to obtain a correlation, at the minimum, three products differing in *in vivo* and *in vitro* are required. With less than three products, only rank order relationship can be obtained. If there is no difference in *in vivo* parameters, only association can be obtained.

In earlier studies, Shah *et al.* (31, 32) conducted two studies (single and multiple dose) using slow-dissolving and fast-dissolving phenytoin sodium capsule preparations. Each of these products were tested utilizing healthy volunteers and epileptic patients. A good correlation was obtained with the *in vitro* dissolution rates and *in vivo* parameters for each of the two phenytoin sodium capsules (32). On the basis of these studies, *in vitro* experimental specifica-

tions were recommended for fast and slow-dissolving phenytoin sodium capsule products which were shown not to be interchangeable because of significant differences in their dissolution behavior and *in vivo* performance (32).

Utilizing a dissolution simulator, Yau and Meyer (33) obtained *in vivo-in vitro* correlations for eleven phenytoin formulations. These workers found the dissolution of the majority of the phenytoin capsules to be quite rapid, with all but three of the eleven products being at least 85 % dissolved within ten minutes. Among these eight capsule products a good *in vitro* and *in vivo* correlation was found. The product with the slowest dissolution profile exhibited the poorest *in vivo* bioavailability (33).

In another study involving hard gelatin capsules, an *in vivo-in vitro* correlation was obtained using seven tetracycline HC1 products (34).

A correlation between the dissolution rate and bioavailability for five indomethacin capsules was investigated in human subjects (35). A good correlation between the *in vivo* parameters, such as AUC, t_{max}, C_{max}, and *in vitro* dissolution rates were obtained using the paddle method at slow stirring rates. This emphasizes that the choice of conditions of *in vitro* test methods is one of the important factors in obtaining good *in vitro-in vivo* correlations.

These are some of the examples of gelatin encapsulated products where a good *in vivo-in vitro* correlation for immediate release products was observed. It is important to emphasize that it is not easy to obtain the correlation. It requires products with varying *in vivo* performance and also *in vitro* methodology that can differentiate between the products. There may be cases, where no correlation has been observed, i.e., where products differ in *in vivo* but not in *in vitro* and vice versa.

The *in vitro* and *in vivo* dissolution of a drug from stressed gelatin capsules

The adverse effect of prolonged storage on *in vitro* disintegration and on subsequent drug release from gelatin capsules has long been known (36). Changes in *in vitro* dissolution due to exposure to high humidity have been observed in capsules containing chloramphenicol (37, 38), tetracycline (38), nitrofurantoin (39), and either water-insoluble or relatively water-soluble agents (16). The principal concern is 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 better indication of stressed gelatin capsule performance *in vivo*.

It has been shown recently that hard gelatin capsules tested in a dissolution medium containing enzymes such as pepsin and pancreatin would negate the effects of adverse storage conditions (such as high humidity and temperatures, and also severe light conditions) on the *in vitro* dissolution performance of capsules (25). Murthy *et al.* (25) have demonstrated that the inclusion of gastrointestinal enzymes may better simulate the physiologic conditions that an orally administered capsule would encounter during its transit through the GI tract. More recently, Dahl *et al.* (28) have shown that there was no change in the *in vitro* dissolution (deionized water as a medium) performance of gelatin-coated acetaminophen (p-acetaminophenol) tablets which were stored up to seven months at room temperature. When these gelatin-coated tablets, however, were stored in the presence of high humidity for 3.5 and 7 months, a significant reduction in both the amount of drug released and standard deviations at each time point were observed (28). In contrast to the above observations, when the gelatin-coated acetaminophen tablets stored in a humidity chamber for 7 months were tested in a 1 % aqueous pancreatin solution, they exhibited the same dissolution profile as tablets stored for seven months at room temperature and also freshly prepared (28) gelatin-coated tablets.

In a series of studies, Mohamad *et al.* (11, 12) have demonstrated that the partial insolubilization of the gelatin shell, observed *in vitro* after storage of hard gelatin capsules for 48 months, did not occur *in vivo*. More specifically, these workers showed that while the rate of the *in vitro* dissolution (in media containing no enzymes) of tetracycline hydrochloride from hard gelatin capsules stored for 48 months at ambient temperature was decreased, no differences in the bioavailability of the antibiotic were observed *in vivo* (11, 12). Similar results were observed by these investigators with hard gelatin capsules containing ampicillin trihydrate (40). The time required for 50 % of ampicillin trihydrate to dissolve from hard gelatin capsules stored for 42 months at room temperature was significantly increased when compared to dissolution times of the antibiotic from freshly prepared capsules. The two batches, however, exhibited comparable bioavailabilities and were found to be bioequivalent when tested in a clinical study involving six human subjects (40).

In an elegant study, Dey *et al.* (29) demonstrated that stressed (high humidity, 75 % RH, and tempera-

ture, 40 °C) hard gelatin capsules containing 200 and 300 mg of etodolac (a non-steroidal anti-inflammatory/analgesic drug) which failed *in vitro* dissolution tests in phosphate buffer (pH 7.5), met dissolution specifications (not less than 85 % drug release in 30 min) when tested in phosphate buffer (pH 7.5) containing 1 % w/v pancreatin. Furthermore, the rate and extent of absorption of the drug from the stressed 200 and 300 mg etodolac capsules in dogs were equivalent to capsules stored at room temperature that passed *in vitro* dissolution tests. Interestingly, the bioavailability of etodolac from 300 mg stressed capsules that failed the dissolution specifications was shown to be equivalent to control capsules (fresh, packaged) in 24 adult male volunteers (29).

The aforementioned studies performed on stressed hard gelatin capsules were overly discriminatory and do not reflect the *in vivo* performance of the product. On the other hand, dissolution of stressed capsules in the presence of enzymes is fairly rapid, nearly the same as in pre-stress conditions, and is a reflection of the *in vivo* behavior of the product.

Test methods

In general, the characteristics of a dosage form are determined by a number of factors such as the nature of the drug, its excipients, packaging form (23), and storage conditions to which it is subjected (41). Each aspect must be weighed carefully when investigating a dissolution problem with the dosage form.

As mentioned earlier, shell disintegration problems can arise due to interactions between the gelatin shell and other chemical components within the capsule shell. These interactions should be separated from drug-capsule wall interactions. It is possible, for example, to carry out diagnostic tests for gelatin/fill interactions by stress-storing the final product and separately testing the gelatin shell from the fill. Additionally, hard gelatin capsules can be emptied after subjecting them to stressed conditions, and then refilled with fresh drug blend. The subsequent dissolution tests could reveal the effect of any possible capsule-fill interactions on the dissolution of the second fill. The classic example of capsule-excipient interaction is from formaldehyde contamination of excipients, for which sensitive detection methods are available (24).

In the case of all types of oral dosage forms there exist concerns regarding the ability of *in vitro* tests to predict *in vivo* performance. From the above discussion, it appears that for hard gelatin-encapsulated dosage forms, the current *in vitro* dissolution tests on stressed-stored products tend to underestimate the *in vivo* dissolution rate of a drug. Hence, critical planning is needed when choosing the *in vitro* dissolution and storage stability testing methods. For gelatin-containing dosage forms, the absence of digestive enzymes from the *in vitro* test medium is an important shortcoming (25, 28, 29), giving artificial negative results concerning the role of the gelatin envelope. In fact, serious doubts have been raised as to whether a dissolution test conducted in an enzyme-free medium can provide meaningful results (41) with regard to the *in vivo* performance of gelatin-encapsulated product. Additionally, simulation of long-term storage tests utilizing high temperature/humidity conditions, although necessary, can produce misleading results if they are not well validated. These conditions could trigger reactions within the gelatin shell, or between the gelatin shell and its contents, which would not occur at lower temperature. In this connection it should be noted that the initial water content of the gelatin shell or the gelatin formulation must be accurately measured. Loss of moisture content from the gelatin dosage form may have serious repercussions on the dissolution profile of the drug it contains. Recently, a sensitive technique which is rapid and non-invasive to the capsules utilizing near infrared methodology has been successfully applied in these laboratories for the determination of moisture content in hard gelatin capsules (42).

Conclusions

1. The advantages and limitations of gelatin-containing dosage forms are well-described in the literature. Crosslinking phenomena, occurring in stressed hard gelatin capsules, and gelatin-coated tablets, could cause considerable changes in the *in vitro* dissolution profiles of drugs. However, in a few limited cases identified in the literature, the bioavailability of the drug from the stressed capsules is not significantly altered when compared to that obtained from freshly packed capsules.

2. It is important to consider the interactions that occur by the presence of certain chemical entities which may contribute to the occurrence of gelatin crosslinking in the gelatin shell of a dosage form. Thus, careful attention should be paid to the purity and chemical reactivity of all of the excipients that are to be encapsulated in a gelatin shell. This is particularly true with soft gelatin capsules, which are considerably more prone to crosslinking phenomena than hard gelatin capsules, since their shells usually contain added materials such as plasticizers and preservatives.

3. It is suggested that *in vitro* dissolution tests of hard gelatin-containing dosage forms be conducted in two stages, one in a dissolution medium without enzymes and secondly in dissolution media containing enzymes (pepsin, at pH 1.2 and pancreatin, at pH 7.2, representing gastric and intestinal media, respectively) (43) prior to *in vivo* evaluation. It is suggested that such *in vitro* tests may constitute a better indication of the *in vivo* behavior of gelatin-encapsulating formulations.

4. Testing for contamination with formaldehyde as well as low molecular weight aldehydes should be a standard part of excipient evaluation procedures.

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