Determination of Extent of Formaldehyde-Induced Crosslinking in Hard Gelatin Capsules by Near-Infrared Spectrophotometry

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Purpose. To predict the degree of crosslinking from formaldehyde-stressed hard gelatin capsules (HGCs) using near-infrared spectrophotometry (NIR).

Methods. HGCs were exposed to a 150 ppb atmosphere of formaldehyde for 2.25, 4.60, 9.42, 16.0 and 24.0 hours. The capsules were filled with fresh amoxicillin, placed in a 90° conical reflector cone, and scanned in a NIR spectrophotometer. Principal component regression (PCR) was employed to analyze the spectra of the intact capsules. Dissolution profiles were then obtained for each experimental group.

Results. The dissolution of amoxicillin from the capsules at pH 1.2 was found to decrease with increasing time of exposure to the formaldehyde atmosphere. A set of principal components (PCs) was formed by a linear combination of the absorbance values at each wavelength scanned. A good correlation was established (r^2 = 0.963) when PC values from the NIR spectra of the HGCs were regressed against percentage of amoxicillin dissolved at 45 minutes, at pH 1.2. Water content of the capsules was found to be the largest determinant in the variation between HGC spectra at each exposure time.

Conclusions. NIR spectrophotometry, combined with PCR, was successful at not only predicting dissolution of HGCs exposed to formaldehyde, but also at determining which wavelengths contributed most to spectral variation of these stressed HGCs.

KEY WORDS: gelatin; crosslinking; formaldehyde; dissolution; near-infrared spectrophotometry.

INTRODUCTION

Gelatin is valuable to the pharmaceutical industry because of the variety of formulations that can be incorporated into the hard gelatin capsule (HGC) and soft elastic gelatin capsule. Gelatin capsules are useful due to their strong yet flexible backbone, polished appearance, ability to hold dyes, and their solubility in aqueous solutions (1).

The susceptibility of gelatin to chemical modification is well known. Of the variety of reagents capable of interacting covalently with gelatin, formaldehyde has been studied most extensively (2). Indeed, crosslinking of gelatin with formaldehyde has been used to produce enteric hard and soft capsules (1–4). However, when gelatin capsules intended for immediate release of their contents are exposed to trace levels of formaldehyde, the effect on in vitro dissolution rates may be adverse (1,5). For example, literature indicates that corn starch, a common pharmaceutical excipient, may incorporate a small amount of hexamethylenetetramine stabilizer (6–9), which upon hydrolysis, forms ammonia and formaldehyde (10).

Current research indicates that reduced in vitro dissolution rates, rather than decreased in vivo bioavailability of drugs, are the principal consequence emanating from the crosslinking of HGCs with low levels of formaldehyde (1,5). This phenomenon has been investigated by several workers (11–14), who utilized carbon-13 nuclear magnetic resonance (^{13}C NMR) and ^{13}C-enriched formaldehyde to show that gelatin reacts with formaldehyde through initial formation of amine methylols (carbinolamines) on lysine and arginine residues, with subsequent production of methylene bridges (crosslinks) between lysine and arginine (11–14). More importantly, the same studies showed that pancreatin, a proteolytic enzyme present in the gastrointestinal tract, can depolymerize crosslinked gelatin (11).

It has become evident that a nondestructive, noninvasive technique suitable for the detection of HGC crosslinking with low levels of formaldehyde could be of vital importance to the pharmaceutical industry. The present work introduces a novel concept of monitoring the extent of formaldehyde-induced crosslinking in HGCs by near-infrared (NIR) spectrophotometry, pharmaceutical applications of which include the determination of water uptake in tablets (15) and hard gelatin capsules (16), detection of tampering in capsules (17) and tablets (18), and analysis of degradation products (19). In the present study, the percent of amoxicillin dissolved at 45 minutes was used as a reference to correlate the degree of crosslinking with data obtained by NIR, which predicted dissolution of the drug from intact HGCs.

MATERIALS AND METHODS

Materials

The dissolution medium consisted of 0.2% w/v sodium chloride and 0.7% v/v conc. HCl in distilled water. Gelatin capsules (size 2, clear, Lot No. 026539) were provided by Capsugel, Greenwood, SC. Formaldehyde, 37 wt% solution in water (stabilized with 15% methanol) was purchased from Aldrich, Milwaukee, WI. The formaldehyde reagent consisted of an aqueous solution of 2.0 M ammonium acetate (98%, Aldrich) and 0.020 M 2,4 pentanedione (99+%, Aldrich), adjusted to pH 6.0. The amoxicillin reagent consisted of 464 ml of 0.10 M citric acid and 536 ml of 0.20 M disodium hydrogen phosphate. The pH of this reagent was adjusted to 5.20 with the either pure phosphate or citric acid buffer. Fifteen ml of 0.0158 M copper sulfate pentahydrate (98%, Spectrum, Gardena, CA) was diluted to a volume of 1L with the pH 5.20 citric acid-phosphate mixed buffer. Amoxicillin trihydrate used for filling the stressed HGCs was Amoxil® (SmithKline Beecham, Philadelphia, PA) brand formulation, while amoxicillin trihydrate (99%) utilized for preparation of standards was obtained from Sigma, St. Louis, MO.

Instrumentation

The fluorescence spectrophotometer used was a Hitachi model F-2000 (Hitachi, Tokyo, Japan) with a 150 W xenon short arc lamp. Fluorescence excitation and emission were at

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410 and 510 nm, respectively. A Vanderkamp® 600 (VanKel Industries, Inc., Edison, NJ) six spindle dissolution tester was used to conduct dissolution tests. Apparatus II was used and the paddles rotated at 50 rpm. The solutions were maintained at 37°C. The NIR spectral data were collected by a Flex® (Bran + Luebbe, Elmsford, NY) spectrophotometer, which is a 19-element filter instrument. Reflectance values were obtained from capsules in a conical reflector (16) at each of the 19 filter wavelengths over a range from 1445 to 2348 nm using a 10 nm filter bandpass. The data were collected with an IBM PS/2 model 50 (International Business Machines, Armonk, NY) computer. Data analysis was performed with a Pentium (133 MHz) personal computer with software written in Speakeasy IV Eta (Speakeasy Corporation, Chicago, IL). The UV-visible spectrophotometer used was a Cary 2200 (Varian, Palo Alto, CA) with a 1.0 nm bandwidth and a 320 nm wavelength.

Procedure

To remove the possibility of significant chemical interaction between active drug (amoxicillin) and formaldehyde, the HGCs in this study were stressed empty rather than filled. Thus, twelve empty, unopened HGCs were exposed to a formaldehyde atmosphere at ambient temperature by placing them on an aluminum wire screen support inside a vacuum desiccator (2.640 L) containing no desiccant. The vacuum port was fitted with a rubber septum to allow introduction of formaldehyde and sampling of the air above the capsules. Exactly 1.0 μL of a solution of 37% formaldehyde in water was introduced into the desiccator chamber. Exactly one hour after formaldehyde introduction, 2.00 ml of the desiccator atmosphere was sampled through the vacuum port with a syringe (Hamilton, Reno, NV). The contents of the syringe were immediately introduced into 2.00 ml of 2,4 pentanedione reagent (20). The resulting solution was incubated at 37°C for 1 h and subsequently cooled to ambient temperature. Fluorescence was determined on the latter solution in a 1.00 cm cuvette. Fluorescence was likewise determined on standard solutions (0–400 ng/ml) of formaldehyde in distilled water, with 2.00 ml standard added to an equal volume of reagent prior to incubation. At 2.25 h after introduction of the formaldehyde into the desiccator chamber (1.25 h after atmosphere sampling), the gelatin capsules were removed from the formaldehyde chamber and were allowed to remain at ambient conditions for 72 h. Four additional experiments were conducted whereby HGCs were exposed to formaldehyde vapors for longer times: 4.60, 9.42, 16.0, and 24.0 h. The chamber atmosphere was always sampled 1 h after formaldehyde introduction. Upon removal of the HGCs from the chamber, they were filled with fresh amoxicillin formulation (Amoxil®). As a control, six fresh, unstressed HGCs which had not been exposed to formaldehyde vapor were also filled with fresh amoxicillin drug blend.

The HGCs were placed in a 90° conical reflector and scanned in the NIR spectrometer. A steel wire support was used to position the capsule vertically within the cone.

The dissolution profile of amoxicillin from the HGCs was determined. Ten mL samples were taken from each dissolution vessel from 0 to 90 min at 15 min intervals, with fresh medium (pH 1.2, 10 mL) replacing the sampled medium. The concentration of amoxicillin in each of the sampled solutions was determined based on the spectrophotometric measurement of a copper-amoxicillin degradation product complex (21). Accordingly, a 0.500 mL aliquot of the dissolution sample was diluted into 5.00 ml of copper sulfate reagent. The resulting 5.50 ml solution was split into two equal portions. One of the two solutions was heated at 75°C for 30 min and, using an ice bath, was cooled to ambient temperature. The optical density of the latter solution was determined spectrophotometrically in a 1.00 cm cuvette, using the unheated solution as reference.

RESULTS AND DISCUSSION

The prediction by NIR spectrophotometry of dissolution rates of drug from formaldehyde-stressed HGCs required a sensitive assay to determine the precise concentration of formaldehyde vapor to which the empty capsules were actually exposed. Hence, a spectrofluorimetric assay (20) was used to ascertain the formaldehyde concentration inside the desiccator chamber which the HGCs were incubated. Regression of formaldehyde standards (0 to 200 ng/ml (ppb)) against their respective fluorescence intensities produced a standard curve (r^2 = 0.992). The formaldehyde concentrations of these standards as analyzed (0 to 200 ppb) represented a range of 0–400 ppb of formaldehyde as prepared, since the latter solutions were diluted with an equal volume of reagent. The fluorescence intensities of the solutions containing the 1 h desiccator-sampled atmosphere from each of the five experiment groups (2.25, 4.60, 9.24, 16.0, and 24.0 h exposure of 12 HGCs to formaldehyde vapor) were determined. From the standard curve, concentration values of the five experiment groups were extrapolated, the mean of which was 145.4 ppb of formaldehyde present in the desiccator atmosphere. This value agreed closely with the theoretical calculation of 151.7 ppb, in which the mass of formaldehyde (400.7 ng) was divided by the volume of the desiccator (2.640 L) into which the formaldehyde was introduced. The determination of formaldehyde in some methods of analysis may be complicated by its existence in both monomeric and polymeric forms. However, the assay utilized in the present work which involves the reaction of one mole ammonia, two moles 2,4 pentanedione, and one mole formaldehyde, requires the latter to be in its monomeric form. Since formaldehyde hydrate (monomer) is consumed in the aforementioned reaction, the equilibrium of formaldehyde in water shifts toward the monomer, as predicted by the law of mass action. Therefore, whether the formaldehyde sampled from the desiccator chamber atmosphere was in polymeric or monomeric form is of little consequence, because the reaction equilibrium assures that all formaldehyde which was present in the aqueous solution of ammonia and 2,4 pentanedione would be available for assay.

In Figure 1, the obtained dissolution profiles (pH 1.2 medium) for amoxicillin from HGCs are shown. With increasing exposure time (up to 16 h) of the HGCs to the 150 ppb formaldehyde atmosphere, the percentage of amoxicillin dissolved at any time point decreased (Figures 1 and 2). Confirming results reported by Carstensen (22) and Chavetz (23), pellicula-tion was observed on all of the formaldehyde-stressed capsules. This thin membrane (pellicle) around the HGC impaired release of amoxicillin from the capsule proper.

Interestingly, the dissolution curves of HGCs, exposed to either 16 or 24 h of 150 ppb formaldehyde, were virtually superimposable (Figure 1). The similarity of the two dissolution curves (16 and 24 h, Figure 1) may be the result of the fact

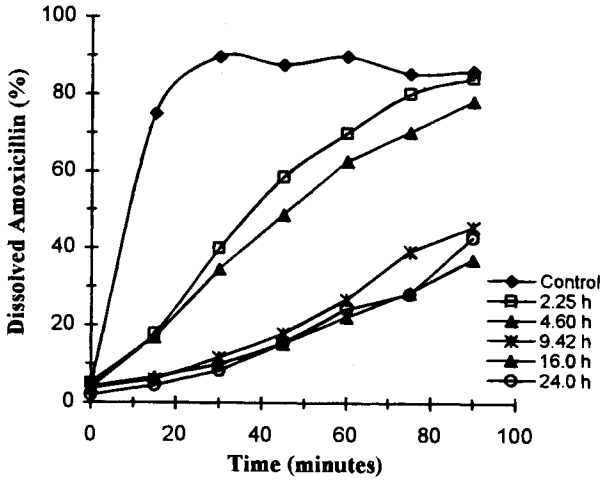

Fig. 1. Dissolution curves of amoxicillin (pH 1.2 medium) from hard gelatin capsules exposed for various times (0, 2.25, 4.60, 9.42, 16.0, and 24.0 h) to 150 ppb formaldehyde atmosphere (ambient temperature). Each point represents the average of six determinations.

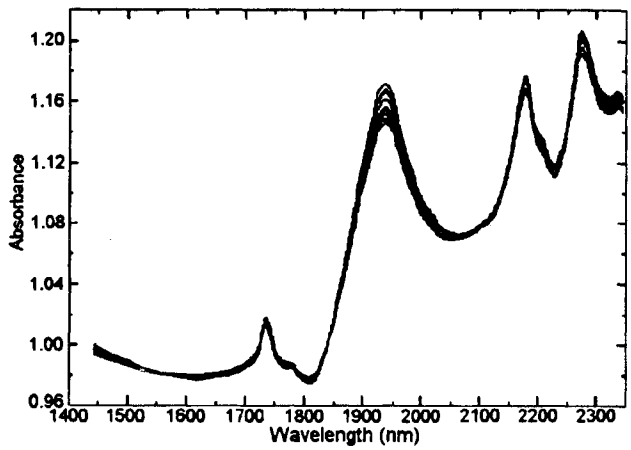

Fig. 3. NIR spectra of 18 HGCs (three for each of the six exposure times: 0, 2.25, 4.60, 9.42, 16.0, 24.0 h) exposed to 150 ppb formaldehyde atmosphere at ambient temperature.

that, given ample time (16 or more hours), the formaldehyde (150 ppb) inside the desiccator was the limiting reagent with respect to the reactive sites, ϵ-amino and guanidino functionalities of lysine and arginine residues, respectively, within the gelatin polypeptide (11–14). Further, the presence of significant amoxicillin in the 90 minute dissolution samples from the HGCs exposed for 16 and 24 h to 150 ppb formaldehyde (~40% amoxicillin dissolved at 90 minutes, respectively, Figure 1) supports the hypothesis that not all of the reactive sites on the gelatin molecule had been modified by formaldehyde (1,11). In general, reaction of gelatin with excessive quantities of formaldehyde produces crosslinked, hydrophobic peptide chains (11) of increased average molecular weight which are no longer soluble in aqueous media and through which drug is not able to migrate.

The NIR reflectance spectra of fresh and formaldehyde-stressed HGCs are shown in Figure 3. These spectra were smoothed by a process in which absorbance values recorded at each of the 19 filter wavelengths were connected by a cubic spline function that passed through each absorbance/wavelength pair (16). Because the NIR spectra contained baseline shifts which were both additive and multiplicative, simple calibration methods were not suitable for data analysis. Instead, principal component regression (PCR) was used to analyze the NIR spectra (24). PCR reduces the dimensionality of a data set, in which there are a large number of correlated variables, by linear transformation into a new set of uncorrelated variables called principal components (PCs). The PCs are structured so that the first few retain most of the variation contained in all the original variables. Thus, the first PC contains information from the constituent which contributes most to the total NIR spectral variation of the data. The second PC is orthogonal to the first and weights most heavily the wavelengths which contribute the most variation to the spectra after removal of the first PC. Progressively smaller contributions to the spectral variation are described by additional, orthogonal PCs. Most of the variation in the NIR spectra (96%) was described by six principal components. Examination of the transformation matrix connecting wavelength and PC hyperspace showed that the signal on the first PC was due to water. The latter has NIR absorbances at 1450 and 1940 nm, and these peaks' intensities were found to decrease with the capsules' increasing time spent in the formaldehyde atmosphere (Figure 3). Since water is a product of many crosslinking phenomena, including that which occurs between formaldehyde and gelatin, the exclusion of water from the HGC shell with increasing time of exposure to formaldehyde (NIR spectra, Figure 3) substantiates the idea that covalent crosslinking is indeed occurring. The first six PCs of the capsule spectra were regressed against percentage of amoxicillin dissolved at 45 minutes from the HGCs exposed to 150 ppb formaldehyde (Figure 4). The NIR spectra of the capsules show a strong linear correlation to actual dissolution of amoxicillin from the formaldehyde-stressed HGCs (Figure 4, SEE (standard error of estimate) = 6.23%, SEP (standard error of prediction) = 7.67%).

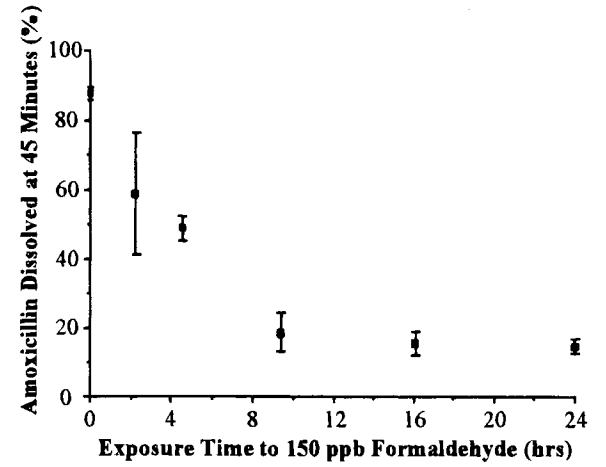

Fig. 2. Dissolution of amoxicillin from HGCs at 45 minutes versus time spent in 150 ppb formaldehyde (ambient temperature).

The dissolution experiments to which the NIR spectra were correlated were conducted in acidic medium because of amoxicillin's superior solubility at low pH. Thus, correlation was established not between the inherent solubility of drug and the NIR spectral data, but rather, between the dissolution from

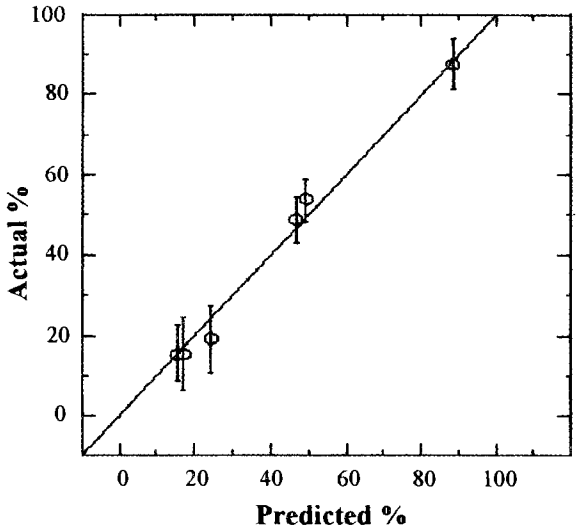

Fig. 4. Correlation of actual dissolution of amoxicillin from HGCs exposed to 150 ppb formaldehyde to dissolution predicted from NIR spectral data. Principal component values from NIR spectra of the HGCs were regressed against percentage of amoxicillin dissolved (pH 1.2 media) from the capsules at 45 minutes (SEE = 6.48%, SEP = 7.67% from cross validation). The cross validation samples are shown superimposed on calibration line, with error bars representing range (extreme values).

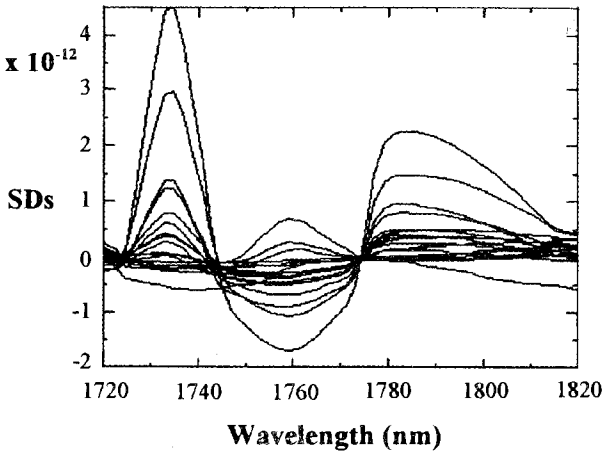

Fig. 5. NIR spectra reconstructed from PCs where crosslinking appears. PCs were eliminated which, after examination of transformation matrices connecting PCs and NIR spectral wavelength, were determined to be due to water absorbance. The y-axis is standard deviation of the formaldehyde-exposed capsules from unstressed, control capsules (0 h of exposure to the formaldehyde atmosphere).

the formaldehyde-stressed capsule shell and its respective NIR spectrum. The effect of acid on the methylene crosslink, which covalently links the ϵ-amino nitrogen of lysine with the guanidino nitrogen of arginine to form an aminal (1), has not been established definitively. Recently, it has been demonstrated, using ^{13}C-NMR and ^{13}C-enriched formaldehyde, that crosslinking of aqueous gelatin solutions does not occur as readily at pH 2 as in neutral (pH 7) or even alkaline (pH 13) media (11). This suggests that the methylene crosslink (aminal) in gelatin behaves similar to an acetal and thus, may be susceptible to acid hydrolysis. Correlation of dissolution data with NIR spectra may therefore have been further improved had dissolution occurred in neutral medium, where the formaldehyde-produced methylene crosslink would be less likely to undergo hydrolysis.

After removal of the first principal component, which was determined by matrix transformation to be due to water, the capsule spectra were reconstructed from PCs where crosslinking appears, with wavelength plotted against standard deviation units (Figure 5). The NIR spectra of fresh control HGCs was selected as the zero standard deviation point on the graph in Figure 5. At 1734 and 1782 nm, most of the spectra of the formaldehyde-stressed capsules deviate from spectra of control (unstressed) HGCs in a positive direction (Figure 5). These spectral features result from new chemical bonds and are concomitant with the formaldehyde-induced crosslinking process in gelatin. Conversely, the capsule spectra show both positive and negative deviations from spectra of unstressed HGCs at 1760 nm (Figure 5) and may be explained by the involvement of an intermediate (carbinolamine) in gelatin crosslinking (1). Specifically, the formation of methylols on amino groups of both lysine and arginine involves a transient carbon-oxygen bond which disappears with the formation of the methylene bridge crosslink. These positive and negative deviations at 1760 nm (Figure 5) of the formaldehyde stressed HGCs (as compared to the unstressed, control group) may be the result of both carbinolamine formation (positive peaks at 1760 nm, Figure 5) and breakdown (negative peaks at 1760 nm, Figure 5), with each HGC spectrum overlay in Figure 5 representing a different point in gelatin capsule crosslinking.

Since IR absorption frequencies (2,500 to 10,000 nm) are well-characterized with respect to functional groups, connection of the NIR frequencies of absorption (1,100 to 2,500 nm) of the formaldehyde-stressed HGCs to IR absorption frequencies would permit a more thorough explanation of gelatin crosslinking mechanisms. This approach, which involves computer-assisted molecular modelling, is currently being investigated in these laboratories.

CONCLUSIONS

The degree of crosslinking, as observed from decreased dissolution rates of amoxicillin from intact hard gelatin capsules exposed to low levels of formaldehyde, was shown to be predictable using NIR spectrophotometry. In this study, the effects of formaldehyde stress storage rather than heat and humidity were investigated with respect to their insolubilzation of the gelatin capsule shell. The importance of studying these two methods of capsule stressing separately lies in the fact that although the end result of gelatin insolubilization may be the same, their respective chemical mechanisms are different (1).

At present, the NIR methods described in this study apply to the stress of HGCs from external/environmental sources. What remains to be seen is if the same technique can be utilized to observe gelatin crosslinking due to formaldehyde contamination of a capsule fill. More specifically, the utility of NIR as a tool to measure crosslinking should be tested on finished capsules, where possible interference from excipients could occur. Accordingly, these laboratories are investigating the effectiveness of NIR spectrophotometry in monitoring the migration of formaldehyde from fill material within a soft elastic gelatin capsule into the capsule shell wall. The latter would

represent a pharmaceutically relevant process of gelatin crosslinking as it pertains to finished product.

When NIR was coupled to principal component analysis, it was possible to distinguish which wavelength(s) contributed most to overall spectral variation of the gelatin capsules. Water content of the capsules was the largest determinant in the variation between HGC spectra at each exposure time, with NIR absorbances of the capsules at 1450 and 1949 nm decreasing with increasing exposure time to formaldehyde. That there are chemical reactions which contribute to the decreased ability of the formaldehyde-stressed HGCs to imbibe water was supported by NIR spectral reconstruction from principal components where crosslinking occurred. The spectrophotometric method described here permits a rapid and nondestructive analysis of intact capsules with or without drug product inside. Using a relatively small set of standards, capsules may be rapidly categorized according to their (NIR) predicted release rate of drug content, and those which fail to meet predetermined criteria may be singled out. Furthermore, the coupling of NIR imaging and laser technologies (25) may permit more efficient, on-line prediction of drug release from dosage formulations.

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