

AN ABSTRACT OF THE THESIS

Ahmad Kh. Bani Jaber for the degree of Doctor of Philosophy in Pharmacy presented on 11, December, 1998. Title: Formulation and *In Vitro-In Vivo* Evaluation of a New Compression-Coated Tablet of Amoxicillin/Clavulanate; and Formulation Potential of the Antimicrobial Peptide Nisin

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Abstract Approved: _____



James W. Ayres

This thesis is comprised of two distinct formulation sections, which are described below:

New compression-coated tablet formulations were developed for amoxicillin/clavulanate. Amoxicillin in an outer coat was separated from clavulanic acid in a core tablet by inactive ingredients in a middle coat. A chewable compression-coated tablet formulation with 50% stearic acid in the middle coat showed high clavulanic acid stability in comparison to the marketed traditional tablet formulation. Stearic acid acted as a hydrophobic barrier that prevents passage of moisture through the outer coats into the core and it helped in the bonding of the outer coats to the core because it softens upon compression. The bioavailability of clavulanic acid, however, was reduced which was attributed to the effect of stearic acid. Stearic acid was removed from the formulation of swallow tablets and replaced with Avicel[®], consequently, the outer coat did not adhere tightly to the core and tended to cap off, which resulted in low stability of clavulanic acid.

In bioavailability studies of the swallow tablets, the two formulations were equivalent to the marketed formulation for amoxicillin, but not for clavulanic acid, which is most likely due to the small sample size studied and high intersubject variation.

Nisin, an antimicrobial protein, was evaluated for ability to emulsify oil-in-water using conductivity measurements. In comparison to Tween[®] 80 and β -casein, nisin showed substantial emulsifying activity. The emulsifying activity was found to be highly concentration- and pH-dependent. Nisin was found to form a gel-like structure at the oil water interface which retarded release of the drug sulfasalazine. Interfacial tension kinetics exhibited by nisin at an oil-water interface were monitored with DuNoy tensiometry. Interfacial pressure kinetics were interpreted with reference to a simple model that allows for a protein to be adsorbed in structurally dissimilar states. The model suggested that nisin's tendency to adapt a more unfolded structure at the oil-water interface increases with decreasing concentration. The effects of nisin on drug release from oil-in-water emulsions, and on erythrocytes were evaluated as well. It was found that nisin retards drug release in emulsions and lyses red blood cells.

FORMULATION AND *IN VITRO- IN VIVO* EVALUATION OF A NEW
COMPRESSION-COATED TABLET OF AMOXICILLIN/CLAVULANATE
AND
FORMULATION POTENTIAL OF THE ANTIMICROBIAL PEPTIDE NISIN

By

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**FORMULATION AND *IN VITRO-IN VIVO* EVALUATION OF NEW
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PEPTIDE NISIN**

CHAPTER 1

INTRODUCTION

With dry compression-coating, water and other solvents in the coating procedure can be eliminated, moisture can be prevented from penetrating into the core, dissolution and disintegration of the tablet can be modified, and incompatible active ingredients can be separated. If a drug tends to discolor readily or tablets develop a mottled appearance because of oxidation or sunlight, these problems can be minimized by incorporating the drug into the core tablet.

Chapters 2 and 3 of the thesis describe compression-coated tablet formulations of amoxicillin and clavulanic acid. The effect of separation of the two active ingredients in the same tablet on their stability to moisture was studied. The effect of incorporating a hydrophobic barrier around the core on stability of clavulanic acid in the core was also studied. The effects of this formulation design on drug dissolution, tablet disintegration, and mechanical strength of the tablet were evaluated and optimized. Based on *in vitro* results, some formulations were selected for further testing in human subjects.

Chapter 4 evaluates and compares pharmacokinetic parameters of a new compression-coated formulation of amoxicillin/clavulanate and Augmentin[®], a reference product. Employing a randomized balanced cross over study, preliminary statistical pharmacokinetic analysis was done for 8 subjects. Data were analyzed by a two one-sided t-test.

Many new chemical entities with promising pharmacological activity never make it through clinical trials because their solubility is so low that a useful drug delivery system can not be developed for them. Emulsions are used effectively to enhance dissolution and bioavailability of hydrophobic drugs and to prevent their precipitation at the site of injection upon parenteral administration. To produce a stable emulsion, relatively large amounts of surfactants are needed, which might have systemic toxicity, such as gastrointestinal irritation and erythrocyte hemolysis. Nisin is a highly surface active cationic polypeptide with antimicrobial activity. It can withstand activity loss during thermal processing, and exposure to acidic environments. These characteristics and others, such as non-toxicity and surface activity, make its potential as an antimicrobial emulsifier attractive in pharmaceutical emulsions.

In Chapter 6, an emulsion conductivity apparatus was used to evaluate the emulsifying activity of nisin as a function of concentration and pH by measuring emulsion stability. For control purposes, all experiments were repeated using Tween[®] 80 and β -casein in place of nisin. Image analysis was used to measure particle size and to characterize emulsion structures.

In Chapter 7, a DuNoüy tensiometer was used to determine how concentration and time affect interfacial activity of nisin at an oil-water interface. Interfacial tension

kinetic data were interpreted with reference to models that allow for nisin to unfold before and after adsorption.

In chapter 8, the effects of nisin on drug release in oil-in-water emulsions, and red blood cells were investigated.

CHAPTER 2

MULTIPLE LAYER COMPRESSION-COATED FORMULATIONS OF AMOXICILLIN AND CLAVULANIC ACID CHEWABLE TABLET

ABSTRACT

Two compression-coated chewable tablet formulations have been developed for amoxicillin and clavulanic acid. The two active ingredients are relatively unstable, particularly clavulanic acid which is rapidly degraded by moisture. Stability of the two active ingredients has been studied in both formulations at room temperature at 96% and 45% relative humidity. Results were compared to those obtained from the marketed formulation and other three formulations which represent a combination of ingredients of the various layers of the compression-coated tablet, but intimately mixed and compressed into a single layer tablet. It has been found that separation of the two active ingredients contributes to enhance the stability of the two active ingredients. The stability of clavulanic acid was optimized by addition of stearic acid into the middle layer of a compression-coated tablet. The dissolution profile of the two active ingredients from the compression-coated tablet was found to be quite different from that of the conventional marketed formulation. This difference is due to the design of the compression-coated tablet. However, both formulations met the USP requirements for dissolution.

INTRODUCTION

Compression-coating tablet consists of a core, on which one or two coats are compressed. The core is formulated as an ordinary tablet using compression or granulation techniques (1). Coating formulations, on the other hand, have some special requirements so that they will make a physically stable tablet (1). They require excellent cohesiveness as well as the ability to adhere to the core. They should be plastic enough to expand slightly with the slight swelling of the core after extrusion of the completed tablet from the die. The maximum size of the granules must be less than the space between the deposited core and the walls of the die so that the granules will readily fill the space. Unlike other coating procedures, such as sugar coating, which may increase a tablet weight by 50-100% of the core weight, compression-coating requires a coat which is about twice the weight of the core (1).

With compression-coating, incompatible substances can be separated by placing one of them in the core and the other in the coating (1). In addition, if a drug tends to discolor readily or develop a mottled appearance because of oxidation or sunlight, these can be minimized by incorporating the drug in the core tablet (1).

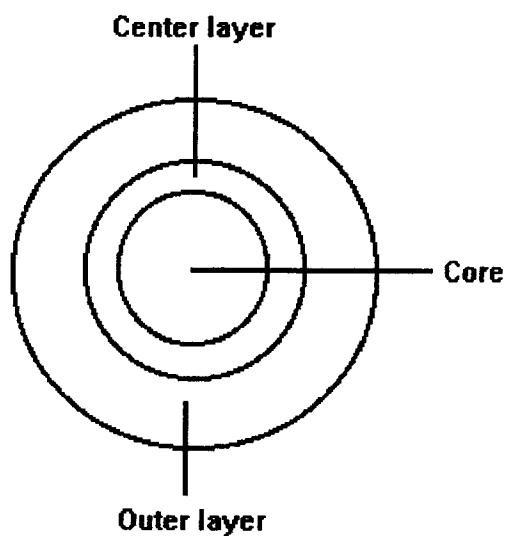
Amoxicillin and clavulanic acid are available as a fixed combination of amoxicillin trihydrate and potassium salt of clavulanic acid in the product Augmentin®. Amoxicillin (α -amino-p-hydroxybenzyl ampicillin) is a semisynthetic penicillin (2). Clavulanic acid (Z-(2R,5R)-3-(β -hydroxyethylidene)-7-oxo-1-azobicyclo-[3.2.0]-heptane-2-carboxylic acid) is a potent inhibitor of the enzyme β -lactamase produced by a variety of Gram positive and Gram negative bacteria (2). Clavulanic acid, however, exhibits only weak antibacterial activity and is therefore unsuitable for use alone (2). The

combination of amoxicillin and potassium clavulanate is active against many β -lactamase producing bacteria which are resistant to amoxicillin alone because clavulanic acid inhibits β -lactamase (2).

Amoxicillin/potassium clavulanate is commercially available for oral administration as film-coated tablets containing a 2:1 or 4:1 ratio of amoxicillin to clavulanic acid, or as a powder for oral suspension or chewable tablets containing a 4:1 ratio of the drugs (2). Commercially available amoxicillin and potassium clavulanate film coated tablets, chewable tablets, and powder for oral suspension should be stored in air tight containers at a temperature less than 24 °C; exposure to excessive light should be avoided (2). Following reconstitution, oral suspensions of amoxicillin and potassium clavulanate should be stored at 2-8°C, and any unused suspension should be discarded after 10 days (2).

The objective of this project is to develop a novel triple compression-coated tablet formulation of amoxicillin/potassium clavulanate. A schematic design of the formulation is shown in Figure 2.1. Both amoxicillin trihydrate and potassium clavulanic acid degrade quickly in solution and the later, in particular, is extremely moisture sensitive and readily discolors (3,4). Potassium clavulanate was formulated as a core tablet, on which two further coats have been compressed. The center layer consists of inert materials which provide complete separation of the two active ingredients. The outer layer contains amoxicillin trihydrate. The destabilization effect of moisture and light on clavulanic acid in the core can be minimized by the two outer coats.

Figure 2.1. Schematic representation of compression-coated tablet.



MATERIALS

Amoxicillin trihydrate (lot # 6453-X5), potassium clavulanate-Avicel mixture (lot # CkA-91), mannitol granular form (lot # B4199), magnesium stearate (lot # TB4236), and sodium saccharin (lot # B4073) were obtained from Biocraft Lab., Fairfield, NJ. Stearic acid powder (triple pressed) (lot # F50335) was from J.T Baker, Philipsburg, NJ. Ac-Di-Sol[®] (modified cellulose gum) (lot # T325) was from FMC, Newark, DE. D&C Yellow # 10 was from Warner Jenkinson Co., St Louis, MO. Artificial banana flavor was from Robert Flavors, South plain Field, NJ.

METHODS

Tablet manufacture

Two compression-coated formulations and three conventional single layer formulations were prepared to study the effect of compression-coated tablet design on the stability of amoxicillin and clavulanic acid, and to achieve a suitable formulation for further dissolution and bioavailability studies. These formulations are based on the composition of four powder mixtures. The compositions of these mixtures are illustrated in Table 2.1.

Mixtures #1, #2, and #3 were prepared by mixing ingredients in a plastic bottle for five minutes with a spatula. Each resulting mixture was passed through a 40 mesh sieve. All ingredients of mixture #3, except mannitol, were mixed well in a plastic bottle for 5 minutes and were sieved through a 40 mesh sieve. Mannitol was added and mixed for 5 minutes to give the final mixture. Mannitol in granular form (direct compression grade) was added last because its granular size was too big to pass through a 40 mesh sieve. Formulations #1 and #2 (Table 2.2) are compression-coated chewable tablets consisting of the same core and outer layer. However, the middle layer consists of stearic acid and Avicel (1:1 mixture) Plus Ac-Di-sol in Formulation #1, or of Avicel plus Ac-Di-Sol in Formulation #2. The purpose of these two formulations is to study the effect of stearic acid as a hydrophobic barrier when incorporated in the middle layer on the stability of clavulanic acid.

Table 2.1. Composition of four mixtures used in different combinations to prepare five formulations of amoxicillin and clavulanic acid.

Mixture #	Ingredient	Amount per tablet (mg)
1	Clavulanic acid	171.2
	Mg-stearate	2.5
	Ac-Di-Sol	7.5
2	Avicel PH 112	50
	Stearic acid	50
	Ac-Di-Sol	5.0
3	Avicel PH 112	100
	Ac-Di-Sol	5.0
4	Amoxicillin	230
	Avicel PH 112	200
	Mannitol	200
	Mg-stearate	5.0
	Na-Saccharine	5.0
	Banana flavor	3.0
	C&D yellow	0.5

Table 2.2. Type and composition of five tested formulations based on mixtures in Table 2.1.

Formulation #	Formulation type	Mixture #1 (mg)	Mixture #2 (mg)	Mixture #3 (mg)	Mixture #4 (mg)
1	Compression- Coated				
	Core	181.2	-	-	-
	Middle layer	-	105	-	-
	Outer layer	-	-	-	743
2	Compression- Coated				
	Core	181.5	-	-	-
	Middle layer	-	-	105	-
	Outer layer	-	-	-	743
3	Conventional	181.2	105	-	743
4	Conventional	181.2	-	105	743
5	Conventional	181.2	-	-	743

Formulations #3, #4, and #5 (Table 2.2) are conventional single layer tablets. Formulation #3 and #4 were prepared from the ingredients combining the core, middle layer, and outer layer of Formulations #1 and #2, respectively. Formulation #5 is a combination of the core and the outer layer of Formulation #1 into a single layer tablet.

The effect of compression-coated formulation on stability of amoxicillin and clavulanic acid can be evaluated by comparing the stability of Formulations #1 and #2 with those of Formulations #3, #4, and #5.

The following procedure was followed in manufacturing Formulations #1 and #2

1. Core

A circular die of 1.1 cm diameter was filled manually with the powder mixture of the core and compressed at 1000 lb using a carver press (Fred S. Carver Inc., Summit, New Jersey).

2. Precompressed core and middle layer

About half of the mixture of ingredients for the center layer was added into a circular die of 1.26 cm diameter. The precompressed core was placed in the middle of the die. The second half of the mixture was added. The mixture with the core inside was compressed manually using a small hand held Vankel press (the exact compression force cannot be determined but it was less than 1000 lb).

3. Final tablet

The mixture of ingredients of the outer layer was compressed around the center layer (from step 2 above) in the same way as previously described using a 1.59 cm wide circular die and 6000 lb to produce the final tablet.

For Formulations #3, #4 and #5, the appropriate amounts of ingredients of each formulation were combined and mixed well for 5 minutes. The final mixture was compressed in the same die used in preparation of the final compression-coated tablets at 6000 Ib.

Stability studies

The tablets of formulations described above were subjected to 96% and 45% relative humidity at ambient temperature for 4 days and 30 days, respectively. Standard all glass aquariums (50 cm long, 26 cm wide, 30 cm high) with plastic cover were used as humidity tanks. Saturated solutions of calcium sulfate trihydrate (provided 96% relative humidity) and potassium carbonate anhydrous (provided 46% relative humidity) were prepared and placed in the bottom of the tanks.

A plastic rack was placed in the tank to hold petri dishes 7 cm above the surface of the saturated solution. Humidity was monitored using a wet and dry bulb (Mason type) hygrometer.

At 97% relative humidity, tablets were removed for determination of amount of intact amoxicillin and clavulanic acid at 6, 12, 24, 36, 48, 60, 72, and 90 hours. Time intervals for amoxicillin and clavulanic acid assay at 45% relative humidity were 1, 3, 5, 7, 9, 11, 13, 15, 20, 25, and 30 days. At each time interval, three tablets of each formulation were assayed for amoxicillin and clavulanic acid content. Each tablet was powdered using a mortar and pestle and the powder was transferred into a 400 ml beaker. 100 ml deionized water was used to rinse the mortar to remove any remaining powder before making up to 500 ml volume. The mixture in the beaker was stirred for 45 minutes

and duplicate 1 ml samples were taken from each beaker. Each sample was diluted with 10 ml distilled water and the solution was vortexed to ensure mixing before preparation of the samples for analysis. A sensitive HPLC method with UV detection (5) was used to assess drug stability

Assessment of formulations

Good flow properties of powders are critical for an efficient tableting operation. When a heap of powder is allowed to stand with only gravitational force acting on it, the angle between the free surface of the heap and the horizontal can achieve a certain maximum for a given powder. This angle is the angle of repose. It was measured for each layer of the powder mixture. The powder was poured through a wide neck funnel and the circular fall pattern traced on paper. The diameter of this circle was measured several times and the mean radius value (r) obtained. The height of the powder pile (h) was measured and the angle of repose (α) was calculated from the following equation (6):

$$\text{Tan } \alpha = h/r$$

Measurements were made in triplicate for tablets, including the core and middle layer tablet.

Hardness of the core, middle layer, and outer layer was determined by a strong Cobb tablet hardness tester. An average of three readings was taken as the final hardness. Recommended hardness limits are greater than 5 kg, although chewable tablets may be somewhat softer (6).

For friability testing, 6 dedusted tablets were weighed, and placed in the laboratory friability tester (VankelKamp). The friabiliator were operated for 100 revolutions. The tablets were dedusted and reweighed.

According to the USP weight variation test, 20 tablets were weighed individually. The mean weight was calculated. The number of tablets outside the 5% limit was determined.

Disintegration

The USP Device with 6 glass tubes 3 inches long, open at the top, and held against a 10 mesh screen at the bottom end of the basket assembly was used to test disintegration. A total of 6 tablets were placed in the apparatus positioned in a 1 L beaker of water at 37 °C. A standard motor device was used to move the assembly containing the tablets up and down at a frequency of 29 cycle per minute.

Dissolution

Dissolution tests were conducted according to the USP XX II paddle method at 37 ° C, 75 rpm. Dissolution media consisted of 900 ml deionized water. The dissolution profile was determined in triplicate for 50 minutes. 5 ml samples drawn via syringe with inline stone filter (2 micron immersible HPLC mobile phase filter, Alltech) were collected at 6, 12, 20, 30, 40, and 50 minutes with replacement of equal volume of deionized water. Amount of each drug released was measured separately using HPLC (5). The undiluted withdrawn solution was used for measuring the amount of clavulanic acid

released, while 1 ml of this solution was diluted with 5 ml deionized water to give a suitable concentration of amoxicillin to be injected into the HPLC.

RESULTS AND DISCUSSION

Stability

Figures 2.2 and 2.3 show that clavulanic acid in Formulation #1 is much more stable at room temperature than clavulanic acid in the marketed formulation and other tested formulations. The most stable formulation of clavulanic acid (Formulation #1) contains stearic acid and Avicel in the middle layer separating the two active ingredients. This layer helps prevent the passage of moisture into the core. Formulation #3 represents a combination of the ingredients of the three layers of Formulation #1 into a single layer tablet. It can be concluded that the compression-coated form with the stearic acid in the center layer separating the two active ingredients results in an increase in clavulanic acid stability.

Photographs were taken for Formulation #1, #5, and the marketed formulation after 24 hours at 96% relative humidity (Figure 2.4) and after 1.83 days at 45% relative humidity (Figure 2.5). Formulations #5 and the marketed formulation were highly discolored as a result of degradation of clavulanic acid. Formulation #1 showed no discoloration in the amoxicillin layer. This visual evidence is consistent with the data depicted in Figures 2.2 and 2.3, indicating that the new Formulation #1 is more stable to the presence of moisture than the marketed formulation.

Figure 2.2. Clavulanic acid stability at 97% relative humidity.

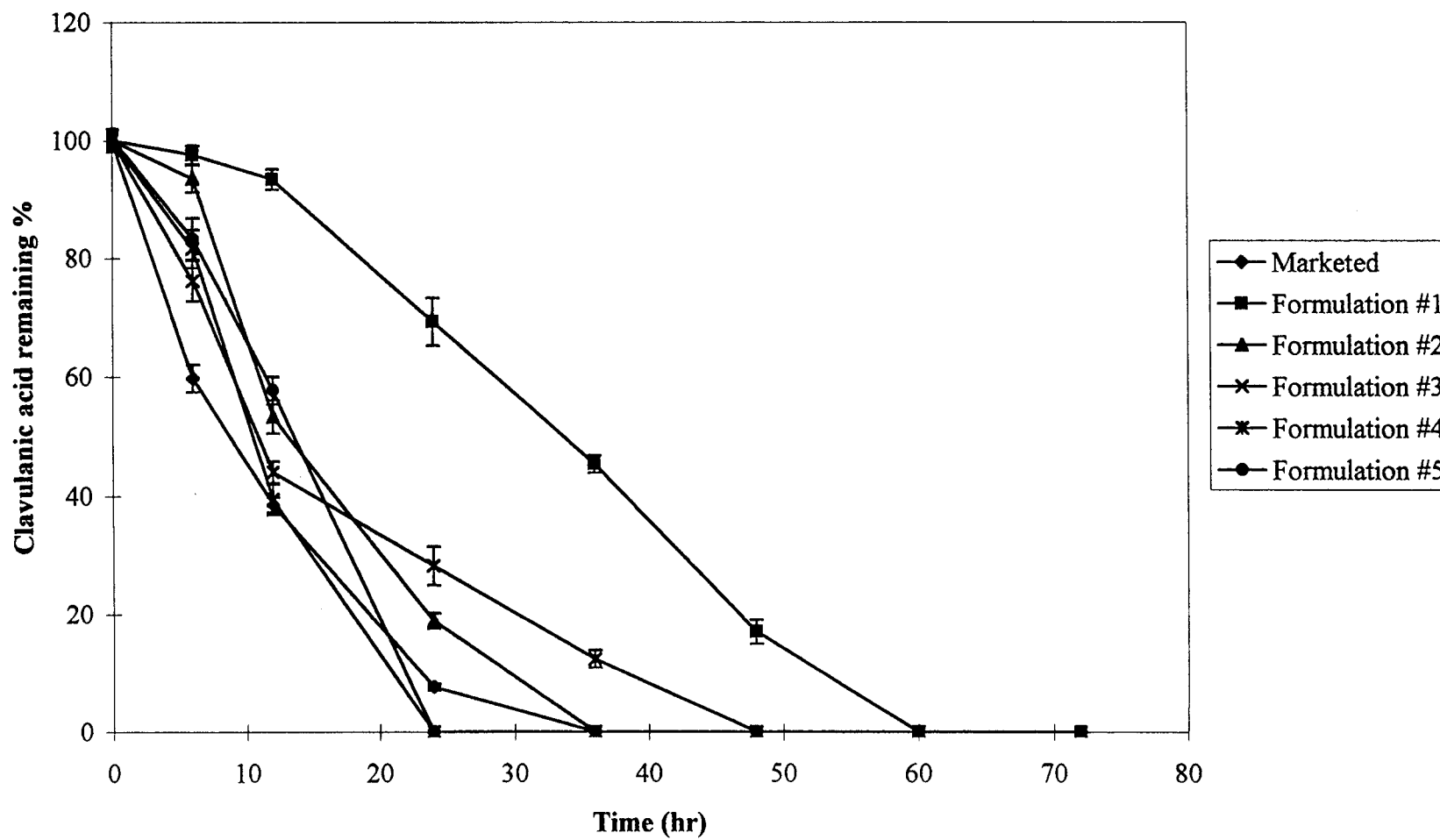


Figure 2.3. Clavulanic acid stability at 45% relative humidity.

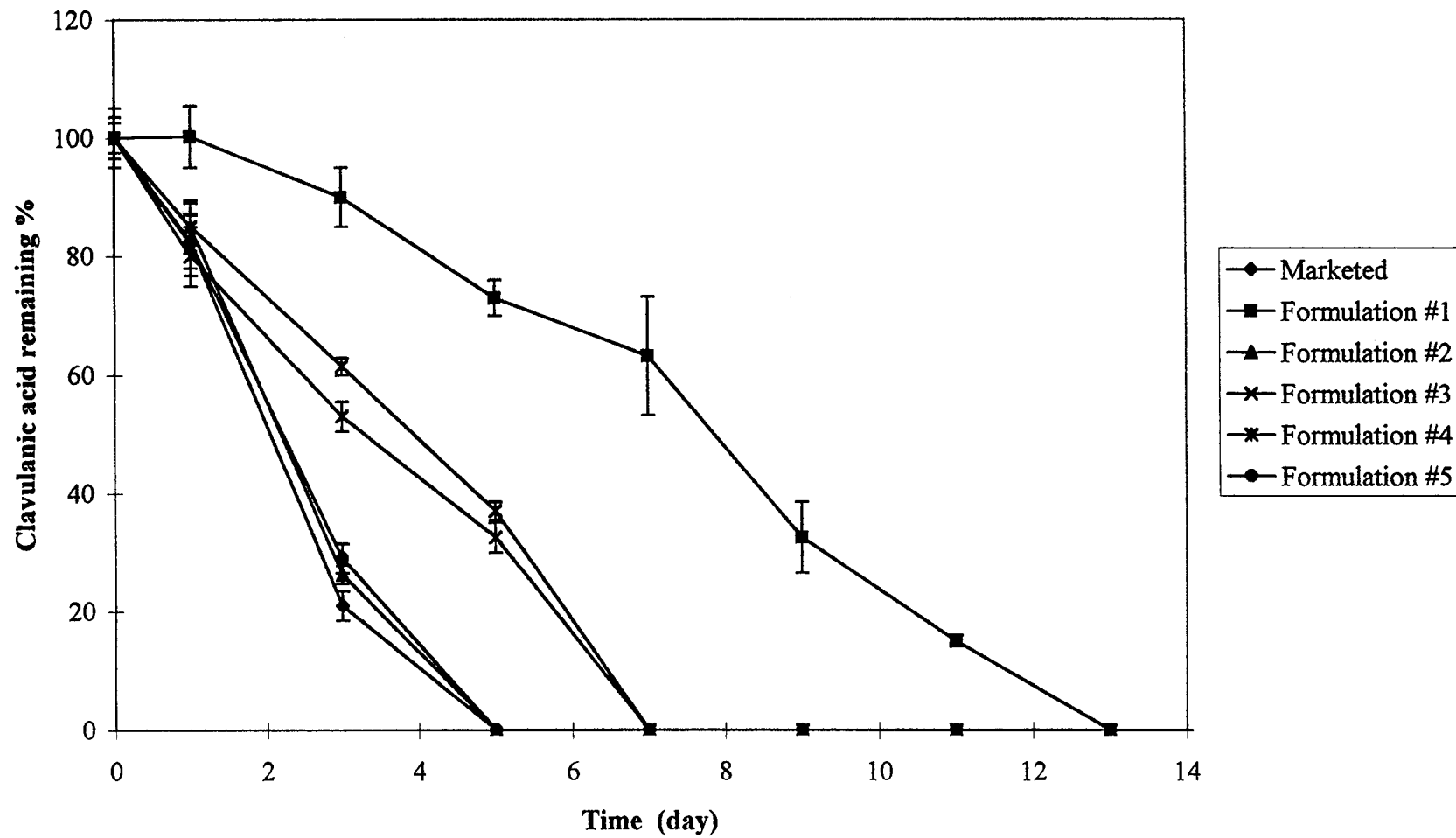


Figure 2.4. Tablets containing amoxicillin and clavulanic acid exposed to 97% relative humidity. Orange color is associated with clavulanic acid degradation. Right: Formulation #1; center: The marketed formulation; left: Formulation #3.



Figure 2.5. Tablets containing amoxicillin and clavulanic acid exposed to 45% relative humidity. Orange color is associated with clavulanic acid degradation. Right: Formulation #1; center: The marketed formulation; left: Formulation #3.



The core of Formulation #1 does discolor as degradation of clavulanic acid occurs, but the degradation is much slower than when clavulanic acid and amoxicillin are intimately mixed.

Figures 2.6 and 2.7 show that amoxicillin is somewhat more stable in Formulations #1 and #2 than in the marketed formulation, and other tested formulations. It can be concluded that the separation of amoxicillin and clavulanic acid is useful to enhance the stability of both amoxicillin and clavulanic acid.

Disintegration and dissolution

Disintegration times for Formulation #1 and the marketed formulation were about 19 minutes and 17 minutes respectively. As shown in Figures 2.8 and 2.9, about 100% of amoxicillin and clavulanic acid dissolved within 30 minutes for both Formulations #1 and the marketed formulation thus meeting the USP requirements which specify that not less than 85% of the labeled amount of amoxicillin and clavulanic acid is dissolved in 30 minutes.

The rate of dissolution of both amoxicillin and clavulanic acid is different for these two products. The rate of dissolution of amoxicillin from Formulation #1 is faster than from the marketed formulation, while clavulanic acid is released at a slower rate from Formulation #1 than from the marketed formulation, particularly during the first 12 minutes of dissolution. This difference was expected due to the different formulation design of the two products.

Figure 2.6. Amoxicillin stability at 97% relative humidity.

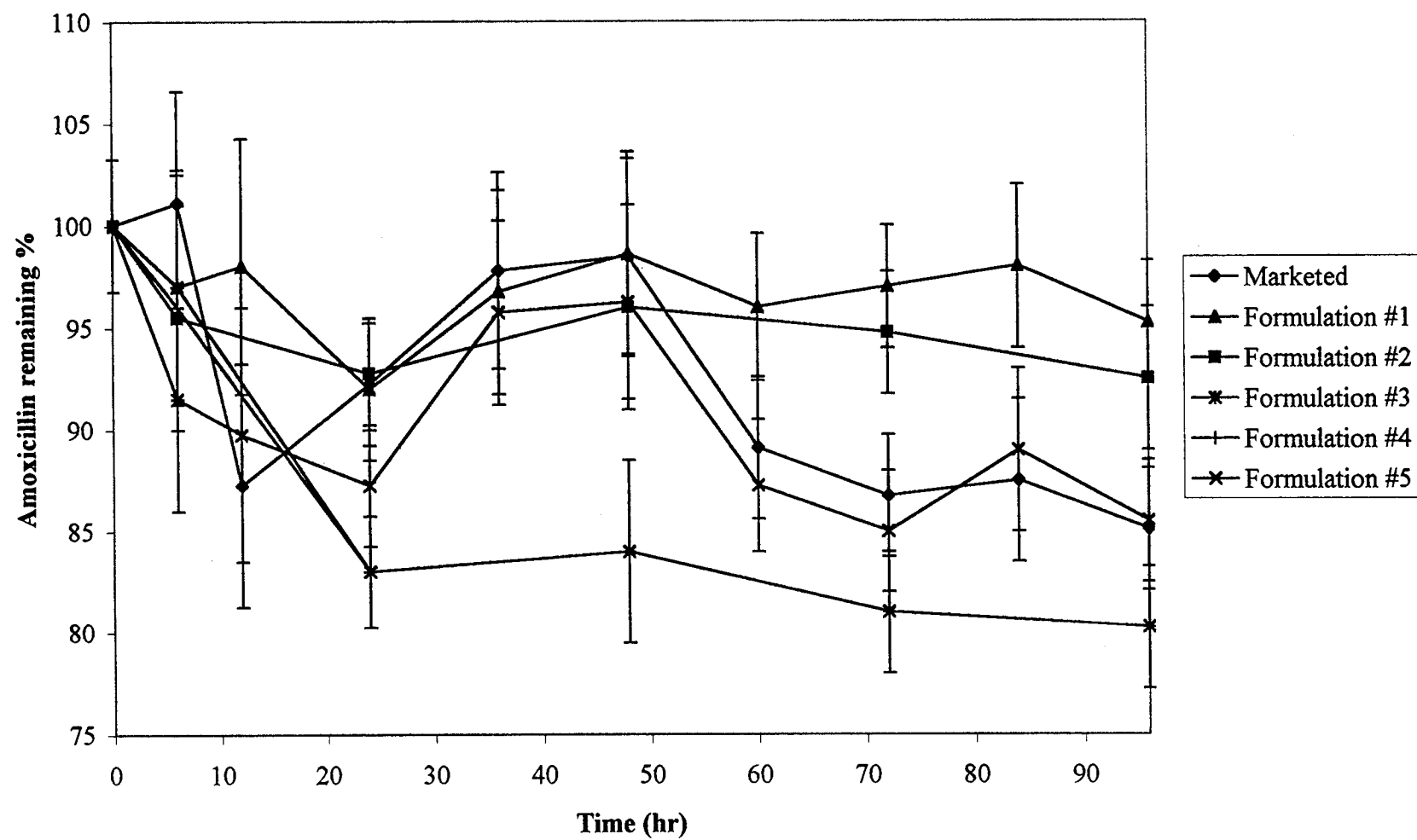


Figure 2.7. Amoxicillin stability at 45% relative humidity.

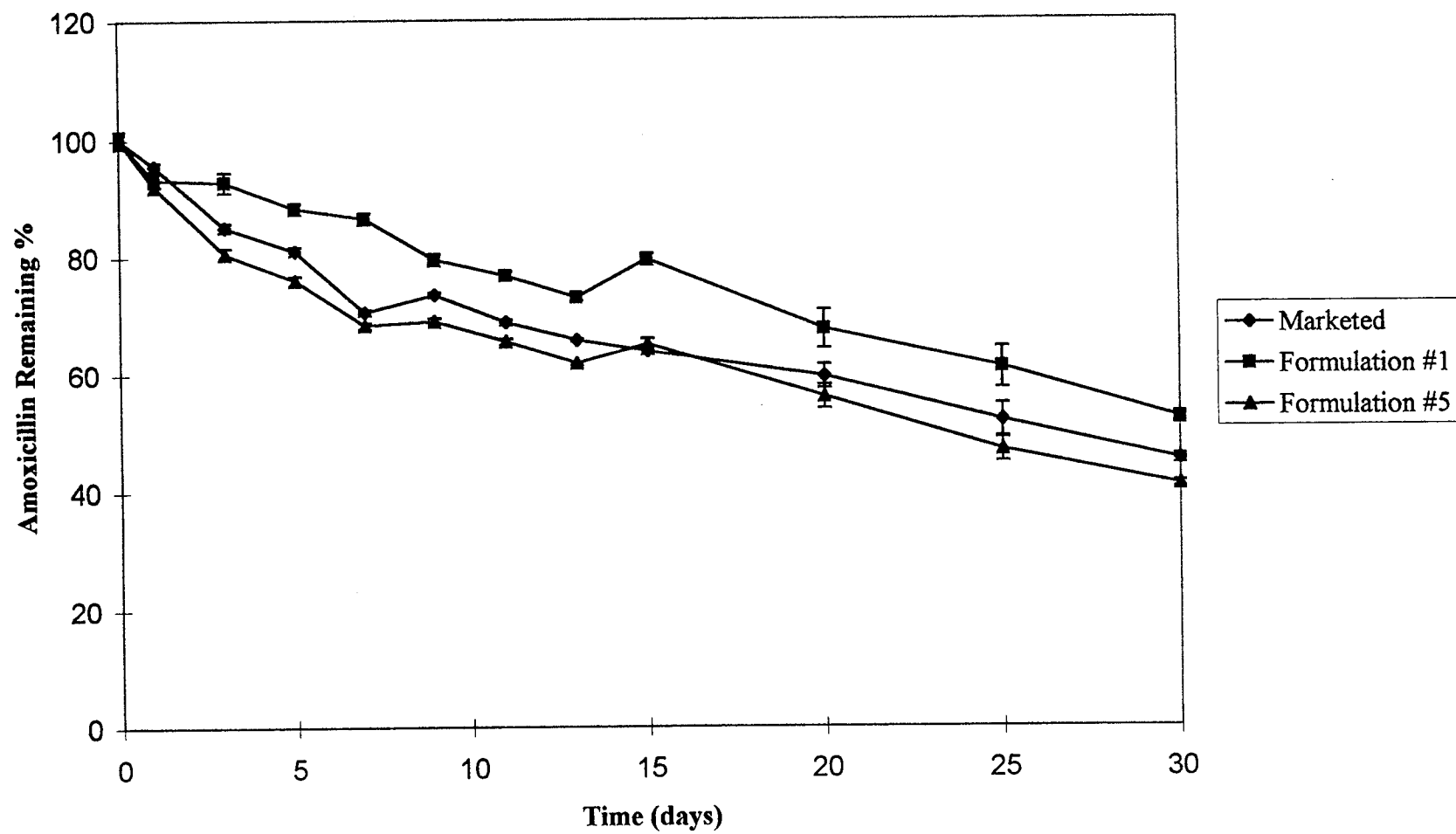


Figure 2.8. Dissolution profile of amoxicillin.

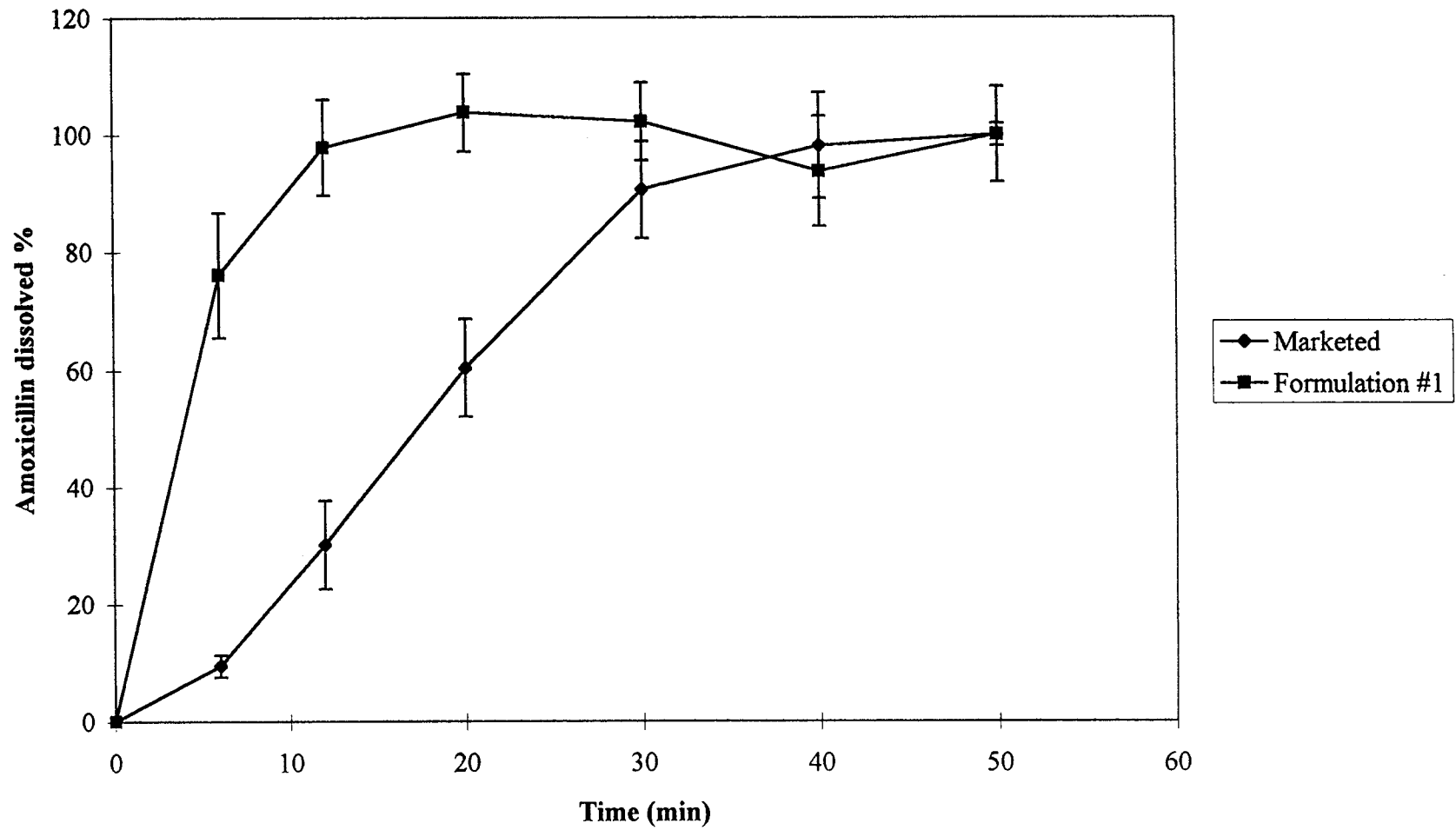
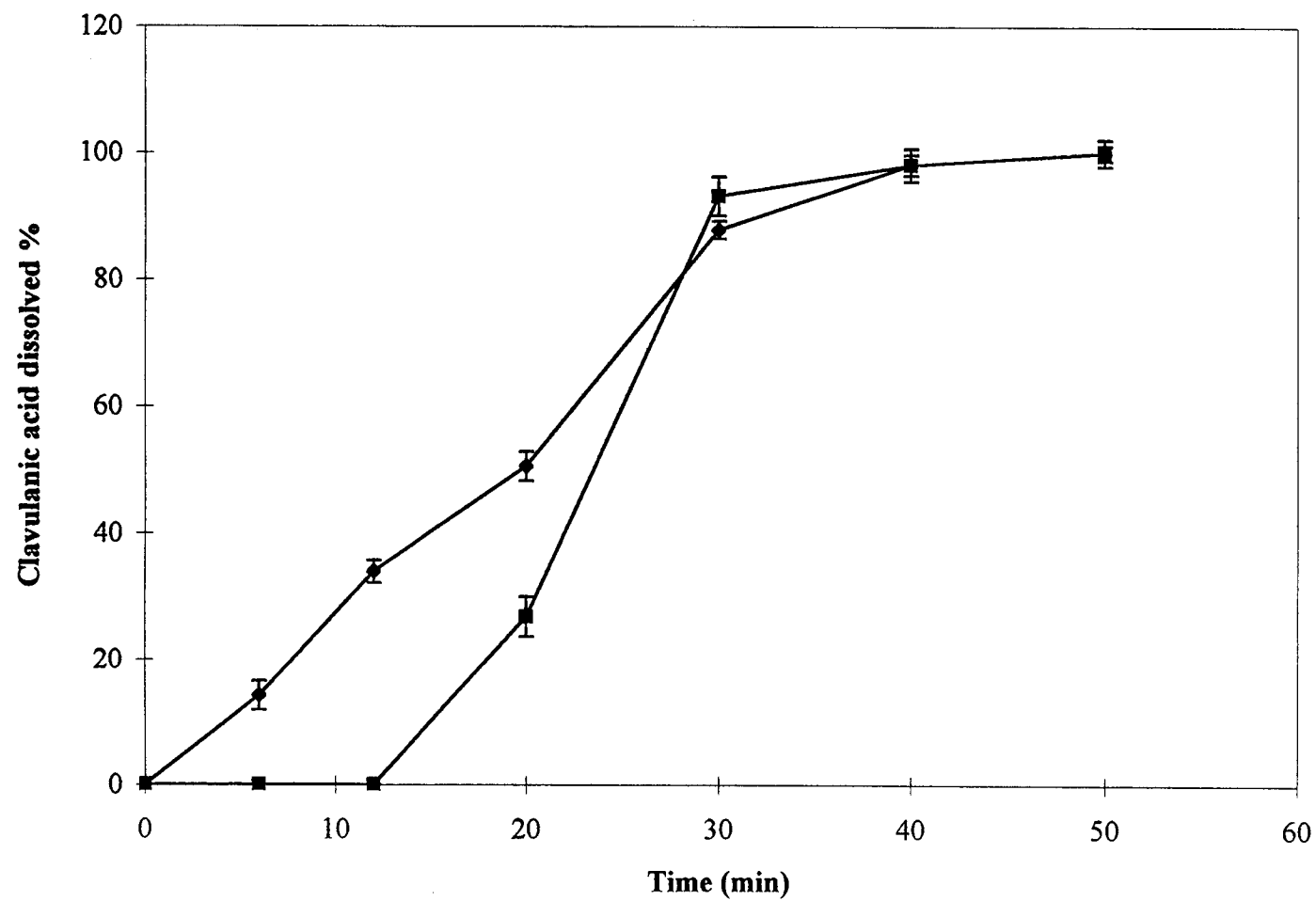


Figure 2.9. Dissolution profile of clavulanic acid.



The marketed formulation is a single layer tablet. Disintegration of the tablet gives an equal proportion of the total granules to be dissolved of both amoxicillin and clavulanic acid during all time intervals. The small difference in dissolution between clavulanic acid and amoxicillin in the marketed formulation can, therefore, be attributed to the difference of solubility and particle size.

The center layer of Formulation #1 is not expected to affect the dissolution of amoxicillin because they are not intimately mixed. However, it may affect the dissolution profile of clavulanic acid because it must disintegrate before clavulanic acid starts to disintegrate. The outer layer of Formulation #1 was designed to disintegrate very rapidly to allow for disintegration of the center layer. Consequently, the dissolution rate of amoxicillin in Formulation #1 was faster than in the marketed formulation.

Retardation of clavulanic acid dissolution during the first 12 minutes was due to the time required for the center layer and the outer layer to disintegrate. Once these two layers eroded, clavulanic acid dissolved rapidly which explains the slight difference of dissolution of clavulanic acid from these two products after the first 12 minutes. The difference in time required for amoxicillin to be completely dissolved between Formulation #1 and the marketed formulation is only 14 minutes, and clavulanic acid dissolution rate is quite similar to the marketed formulation after the first 12 minutes of dissolution.

These slight differences may not significantly affect the absorption rate or extent of both active ingredients. This conclusion is particularly expected in the case of chewable tablets, which are to be chewed before swallowing.

Formulation assessment

Usually, powders of angle of repose less than 30° are described as having good flow properties, while an angle of repose larger than 40° indicates poor flow properties (6). The angle of repose of each of the mixtures used in Formulation #1 (Table 2.3) indicates reasonable flow properties. The three layers of the compression-coated tablets should have good friability in order to withstand handling in a production environment. As shown in Table 2.4, the core and the final tablet have friability values within acceptable range (less than 1% weight loss). All weights of the twenty tablets tested (Table 2.5) were within the 5% limit which meets USP requirements which specify that not more than 2 tablets outside the 5% limit is allowed.

Table 2.3. Angle of repose of the mixtures used in Formulation #1.

Mixture #	Angle of repose
1	27
2	34
3	32

Table 2.4. Thickness, hardness and friability of Formulation #1.

Tablet part	Hardness	Friability	Thickness
	(kg)	(%)	(mm)
Core	4.7	0.34	2.2
core and first coat	*	*	2.3
Final tablet	>15	0.15	5.1

*The tablet press used to make this layer provided low pressure.

Table 2.5. Weight (mg) of finished tablet of Formulation #1.

1033.4	993.63	1026.1	996.94	1016.0
1011.2	1021.4	1034.5	1027.5	980.57
1037.7	989.5	969.87	1007.5	1003.2
993.25	998.5	975.59	989.99	1002.6

CONCLUSIONS

Clavulanic acid is highly moisture sensitive substance, and discolours intensely upon exposure to high humidity. Separation of clavulanic acid and amoxicillin can be achieved in the same dosage form as multiple compression-coated tablet; clavulanic acid in the core can be separated from amoxicillin in the outer coat by inactive ingredients in the middle layer. Stability of clavulanic acid can be maximized by incorporation of stearic acid in the middle layer, which forms hydrophobic barrier and prevents moisture passage into the core. The separation of the two active ingredients results in enhancing the stability of amoxicillin.

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CHAPTER 3

MULTIPLE LAYER COMPRESSED-COATED FORMULATIONS OF AMOXICILLIN/CLAVULANATE SWALLOW TABLETS

ABSTRACT

A compressed-coated swallow tablet formulation of Amoxicillin/clavulanate was developed. The tablet consisted of three parts: core, middle layer (inner coat) and outer layer (outer coat). Amoxicillin in the outer layer was separated from clavulanic acid in the core by inactive ingredients in the inner coat. The outer coat tended not to bind very well to the middle layer and to cap off; the formulation has been optimized to solve this problem. Dissolution profiles of amoxicillin and clavulanic acid from the compressed-coated tablet formulation were similar to those from the marketed single layer formulation. The compressed-coated formulation and the marketed formulation showed similar amoxicillin stability to moisture. However, clavulanic acid was less stable in the compressed-coated tablet formulation than in the marketed formulation, when the two formulations were exposed to high and medium humidity conditions.

INTRODUCTION

A compressed-coated tablet consists of a core on which one or two coats are compressed. Such formulation design can be used to separate active ingredients and to improve drug stability to moisture and light (1).

Amoxicillin, is an aminopenicillin which differs structurally from ampicillin only in the addition of an hydroxyl group on the phenyl ring (2). Amoxicillin is usually bactericidal in action. Concurrent administration of clavulanic acid does not alter the mechanism of action of amoxicillin (2). However, because clavulanic acid has high affinity for and binds to certain β -lactamase that generally inactivate amoxicillin, concurrent administration of the drug with amoxicillin results in a synergistic bactericidal effect and expands the spectrum of activity of amoxicillin against many strains of β -lactamase producing bacteria (2). Amoxicillin and clavulanic acid is commercially available for oral administration as film-coated tablets (2). Each tablet contains 250 or 500 mg of amoxicillin and 125 mg of clavulanic acid. The tablet should be stored in tight containers at temperature less than 24 °C; exposure to excessive humidity should be avoided (2).

The objective of this study is to develop a formulation of amoxicillin/clavulanate as a compressed-coated tablet with clavulanic acid in the core and amoxicillin in the outer coat. This formulation design can prevent mottling of the tablet that results from discoloration of clavulanic acid because of hydrolysis by moisture and oxidation by sunlight.

MATERIALS AND METHODS

Amoxicillin trihydrate (lot # 6453-X5), potassium clavulanate-Avicel mixture (lot # CkA-91), and Magnesium stearate (lot # TB4236), were obtained from Biocraft Lab., Fairfield, NJ. Ac-Di-Sol[®] (modified cellulose gum) (lot # T325) was from FMC, Newark, DE. Sodium stearyl fumarate (Pruv[®]) (lot # 21201X) was from Mendell, Patterson, NY. Acetyltributyl citrate (Citroflex[®]) (lot # N414040) was from Morflex Chemical Co., Greensboro, North Carolina. Eudragit[®] E 30 D (lot # 12851232), Eudragit[®] NE 30 D (lot # 1260812084), Eudragit[®] RS 30 D (lot # 0440218012) and Eudragit[®] RL 30 D (lot # 0440218012) were obtained from Röhm Pharma, Malden, MA.

Tablet manufacture, stability studies and dissolution studies were performed according to reference (3).

RESULTS AND DISCUSSION

Tablet manufacture and formulation

Previous generic formulation of amoxicillin/clavulanic as a compressed-coated chewable tablet with stearic acid in the middle layer showed more stability for clavulanic acid and amoxicillin than the marketed single layer formulation (4). However, the dissolution profiles of the two active ingredients of the generic were slightly different from that of the marketed formulation (4). In addition, bioavailability studies (5) showed that the generic was not bioequivalent to the marketed formulation for clavulanic acid; clavulanic acid T_{max} was delayed and its C_{max} was relatively low for the generic in

comparison to the marketed formulation (5). These differences were attributed to stearic acid, a known inhibitor of gastric emptying, which may delay the absorption of clavulanic acid. Accordingly, stearic acid was removed from the formulation of Amoxicillin/clavulanate oral compressed-coated tablet and Formulation #1 of was prepared and is listed in Table 3.1.

Table 3.1. Formulation #1 of compressed-coated amoxicillin/clavulanate swallow tablet.

Tablet part	Ingredient	Amount per tablet (mg)	Compression force (lb)
Core	Clavulanic acid	312.7	1000
	Ac-Di-sol [®]	5.0	
	Avicel [®] pH112	50.0	
	Mg-stearate	3.7	
Middle layer	Avicel [®] pH112	250.0	2000
	Mg-stearate	2.5	
Outer layer	Amoxicillin	573.9	6000
	trihydrate		
	Avicel [®] pH112	60.0	
	Mg-stearate	6.3	

A schematic representation of the tablet with the dimensions of each layer is shown in Figure 3.1. Each layer showed acceptable friability and hardness. However, the outer layer did not bond very well to the middle layer, as gaps were seen visually in the edges of the final tablet. Scanning electron microscopy of cross sections of the tablet provided further information about how the tablet bonds together. Figure 3.2 shows a triple layer compressed-tablet under high magnifications. Gaps can be seen separating the outer coat and the rest of the tablet and within the outer coat.

Figure 3.1. Schematic representation of amoxicillin/clavulanic acid compression-coated swallow tablet.

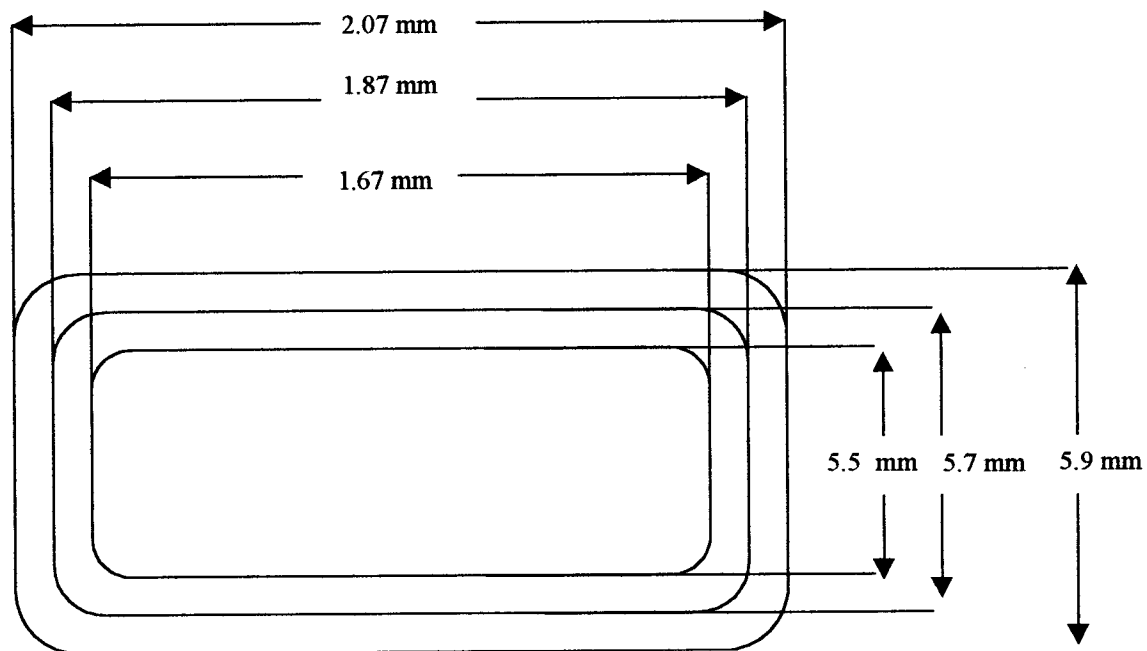
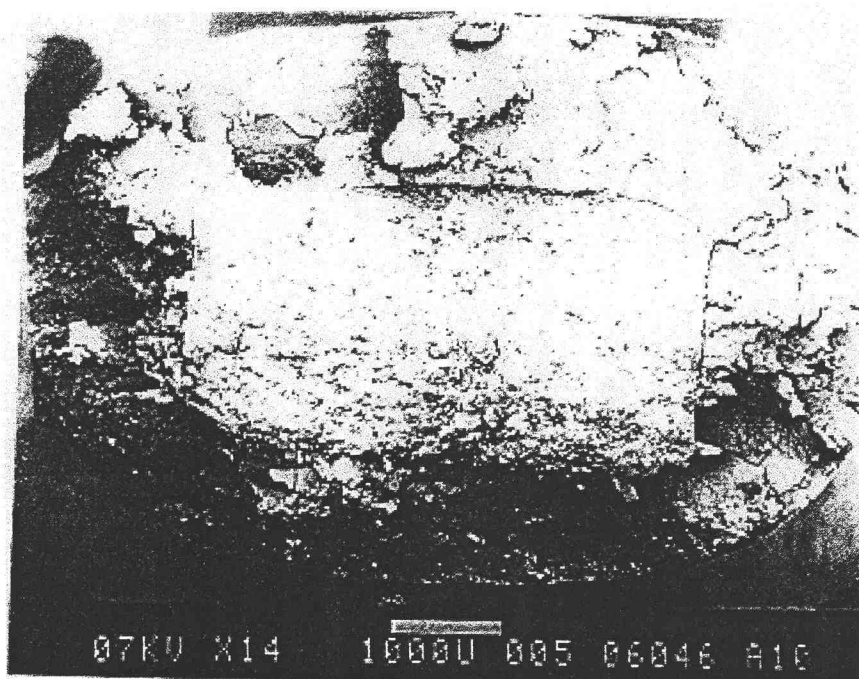


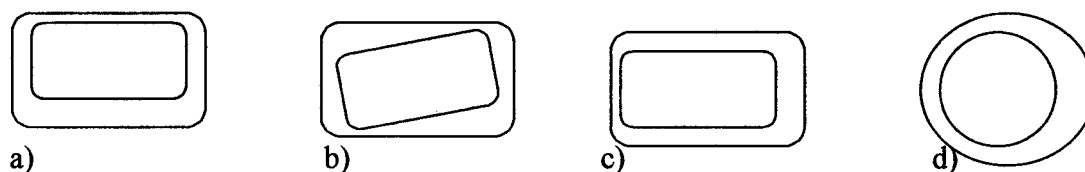
Figure 3.2. Cross section of compression-coated amoxicillin/clavulanic acid swallow Tablet. Magnification, A = X14 and B = X45.



Capping of the outer coat in a compressed-coated tablet

Here are several reasons for capping of the outerlayer in a compressed-coated tablet. The coat might not have a good cohesiveness or ability to adhere tightly to the core (1). The outer coat might not be plastic enough to expand slightly with the slight swelling of the core after the extrusion of the completed tablet from the die (1). The coating may cap off because there is an excess amount of fine powder, glidant, disintegrant or lubricant, since these have little cohesiveness (1). The cores may have been compressed too hard and their surfaces densified so that the coating can not bond. Hard cores tend to be elastic rather than plastic upon release of the pressure; when the core is ejected from the die, the rebound of the core pops the top off the tablet (1). Improper centration of the core either vertically or horizontally produces weak edges (Figure 3.3) (1), and the coat will not hold together.

Figure 3.3. Examples of off-centering. Faults in compression coating: a) unequal coating; b) cocking; (c) and (d) off-center.



Optimization of Formulation #1 to reduce the capping of the outer layer

It is customary to use the same material in the coating as in the core, a practice based on the theory that like substances will bond better to like than to different materials (1). In Formulation #1, the same diluent and lubricant were used in all layers of the compressed-coated tablet. Wolf (6) has recommended that addition of 2% acacia can improve the binding of the coat to the core, and 1.75% of gelatin can impart elasticity for that coat. Acacia and gelatin are hygroscopic substances; they might interfere with the stability of amoxicillin and clavulanic acid, which are moisture sensitive substances (7,8). Thus, they were not investigated.

Metallic stearates can interfere with the bonding of the layers of a compression-coated tablet (1). For this reason, the effect of changing Mg-stearate in Formulation #1 with other lubricants, such as Pruv[®], Lubritab[®] and DL-leucine, on the cohesiveness of the compression-coated tablet was studied by visual inspection of compressed, and often cracked tablets. Among the three lubricants, it was found that the use of Pruv[®] results in fewer and narrower gaps in the edges of the final tablet.

As a way of enhancing bonding between layers in conventional layer tablets, the first layers are compressed at very low compression force and the last layer is compressed into the soft layers at high compression force (1). In compression-coated tablets, the cores are required to be sufficiently hard in order to withstand handling in the transfer devices, and transfer on the coating machine; the core should have good friability and, at the same time, should be soft enough for sufficient bonding with the coat (1). The compression forces used for the core and middle layer in Formulation #1 were the minimum to achieve acceptable friability in the two layers.

In order to achieve formulations for the core, inner coat and outer layer that have an acceptable layer friability at the lowest possible compression force, and have positive effect on the cohesiveness of the final tablet, ingredient changes in the three layers were studied and are listed in Table 3.2. According to Table 3.2, increasing the percentage of Avicel® in the outer layer, inclusion of Emcompress® (dicalcium phosphate) in the core and middle layer, and reducing the lubricant percentage in the outer layer contributes to enhancing the compressibility of the final tablet. In addition, reducing the percentage of lubricant in the core and middle layer improves both layer friability and compressibility of the final tablet. Formulation #1 was adjusted according to Table 3.2 and is listed as Formulation #2 in Table 3.3. Formulation #2 showed less cracking and had fewer gaps in the final tablet than Formulation #1.

One approach which may reduce capping of the outer coat in Formulation #2 is to granulate Avicel® with a nonhygroscopic polymer, that can improve its cohesiveness, adherence to the core and elasticity, before it's addition to the mixture of the outer coat. Acrylic polymer, available under the trade name Eudragit®, are used in coating tablets, capsules and granules for several purposes, such as improving drug shelf life, delaying drug absorption and improving appearance (9). In addition, some types of this class of polymers, such as Eudragit® E 30 D, Eudragit® NE 30 D, Eudragit® RS 30 D, and Eudragit® RL 30 D, are used as binders in powder granulation to improve compression characteristic on tableting (hardness and abrasion) (9). Eudragit® E 30 D and Eudragit® RL 30 D are rapidly disintegrating polymers (9).

Table 3.2. Effect of ingredient changes on layer friability and cohesiveness of compressed-coated tablet of Formulation #1.

Ingredient change	Effect on layer friability ¹	Effect on the cohesiveness of the final tablet ²
Increasing the percentage of Avicel® in the outer layer	No effect	Positive
Replacement of Avicel® in the core with an equivalent amount of Emcompress®	No effect	Positive
Replacement of Avicel® in the middle layer with an equivalent amount of Emcompress®	Negative	Positive
Replacement of Avicel® in the outer layer with an equivalent amount of Emcompress®	No effect	Negative
Replacement of Avicel® in the core with an equivalent amount of CaSO ₄ .2H ₂ O	No effect	No effect
Replacement of Avicel® pH112 in the middle layer with an equivalent amount of CaSO ₄ .2H ₂ O	Negative	Negative
Replacement of Avicel® in the outer layer with an equivalent amount of CaSO ₄ .2H ₂ O	No effect	Negative
Replacement of Avicel® in the core with an equivalent amount of lactose anhydrous	No effect	No effect
Replacement of Avicel® in the middle layer with an equivalent amount of lactose anhydrous	No effect	No effect

Table 3.2 (Continued)

Replacement of Avicel® in the outer layer with an equivalent amount of Lactose anhydrous	No effect	Negative
Reducing the percentage of lubricant in the core	Positive	Positive
Reducing the percentage of lubricant in the middle layer	Positive	Positive
Reducing the percentage of lubricant in the outer layer	No effect	Positive

¹ Based on the compression force required to achieve the same or better layer friability than in Formulation #1: lower force: positive; the same force: no effect; higher force: negative.

² Based on visual inspection of the final tablet.

Table 3.3. Formulation #2 of compressed-coated amoxicillin/clavulanate swallow tablet.

Tablet part	Ingredient	Amount per tablet (mg)	Compression force (lb)	Friability
Core	Clavulanic acid	312.7	500	0.29
	Ac-Di-sol [®]	5.0		
	Emcompress [®]	100.0		
	Pruv [®]	2.1		
Middle layer	Avicel [®]	100	3000	0.54
	Emcompress [®]	250.0		
	Pruv [®]	0.9		
Outer layer	Amoxicillin	573.9	7000	0
	trihydrate			
	Avicel [®] pH112	150.0		
	Pruv [®]	3.6		

Eudragit® NE 30 D, a permeable and swellable polymer, possesses a high binding capacity and elasticity and, therefore, has special opportunity for application in granulation (9). Eudragit® RS 30 D has low permeability that is independent of pH (9). These acrylic polymers were tested for their effect on the compressibility of the final tablet as a granulating agent for Avicel® in the outer layer. The effect of increasing plasticity of the Avicel® granules with Citroflex® on tablet compressibility was also studied.

Table 3.4 Shows that granulation of Avicel® with any of those acrylic polymers, particularly Eudragit® NE 30 D, as well as increasing the plasticity of the granules can minimize the capping of the outer coat without significant effect on disintegration of the final compressed-coated tablet. Because drugs were not involved in this granulation, and disintegration of the final tablet was not prolonged, Avicel® granulation with any of these acrylic polymers in the outer layer will not affect the dissolution of amoxicillin or clavulanic acid.

Based on the foregoing formulation tests, a final formulation of compressed-coated amoxicillin/clavulanic acid swallow tablet was selected as listed in Table 3.5 as Formulation #3. Further stability and dissolution studies were performed on this formulation.

Table 3.4. Effect of granulation* of Avicel® in the outer layer of Formulation #2 with several types of Eudragit® polymers with or without 5% Citroflex® on tablet disintegration and cohesiveness.

Granulation process	Disintegration Time (minutes)	Time after which crack in the outer layer was visually seen (days)
No granulation	6	Immediately
Granulation with Eudragit® E 30 D	6	2
Granulation with Eudragit® NE 30 D	8	3
Granulation with Eudragit® RS 30 D	11	1
Granulation with Eudragit® RL 30 D	13	1
Granulation with Eudragit® E 30 D plus 5% Citroflex®	5	4
Granulation with Eudragit® NE 30 plus 5% Citroflex®	9	6
Granulation with Eudragit® RS 30 D plus 5% Citroflex®	9	4
Granulation with Eudragit® RL 30 D plus 5% Citroflex®	9	2

*12 ml Eudragit® dispersion (30%) with or without 5% Citroflex® were used in granulating 10 gm Avicel®.

Table 3.5. Formulation #3 of compressed-coated amoxicillin/clavulanate swallow tablet.

Tablet part	Ingredient	Amount per tablet (mg)
Core	As in Table 3.3	As in Table 3.3
Middle layer	As in Table 3.3	As in Table 3.3
Outer layer	Amoxicillin trihydrate	573.9
	Avicel [®] pH112 Granulated with	150.0
	Eudragit [®] NE 30 D and Citroflex [®]	
	According to Table 3.5	
	Pruv [®]	3.6

Stability studies

Figures 3.4 and 3.5 illustrate that both the marketed formulation and Formulation #3 show similar stability for amoxicillin at low humidity, however, amoxicillin was slightly more stable in Formulation #3 than in the marketed formulation, which might suggest that separation of clavulanic acid and amoxicillin helps in stabilization of amoxicillin.

The marketed formulation shows more stability for clavulanic acid than Formulation #3 (Figures 3.6 and 3.7). In a previous study, it was found that formulation of clavulanic acid as a compressed-coated chewable tablet with Avicel® in the middle layer had similar stability to that of the marketed single layer chewable tablet formulation. Unlike the chewable tablet, the marketed swallow tablet is coated with three coats: hydroxypropylmethyl cellulose, methylacrylic acid, methylacrylate copolymer and shellac (10). These three coats might provided a barrier to moisture and contribute to enhancing the stability of clavulanic acid in the marketed formulation. The degradation of clavulanic acid in the core of Formulation #3 might be enhanced by cracking in the outer coat upon exposure to moisture; the compressed-coated tablet tends to crack upon exposure to humidity, which will increase the amount of moisture that can reach into the core. It is expected that application of polymer coats over the multiple compression-coated tablet will greatly enhance clavulanic acid stability in the core.

Figure 3.4. Amoxicillin stability at 97% relative humidity.

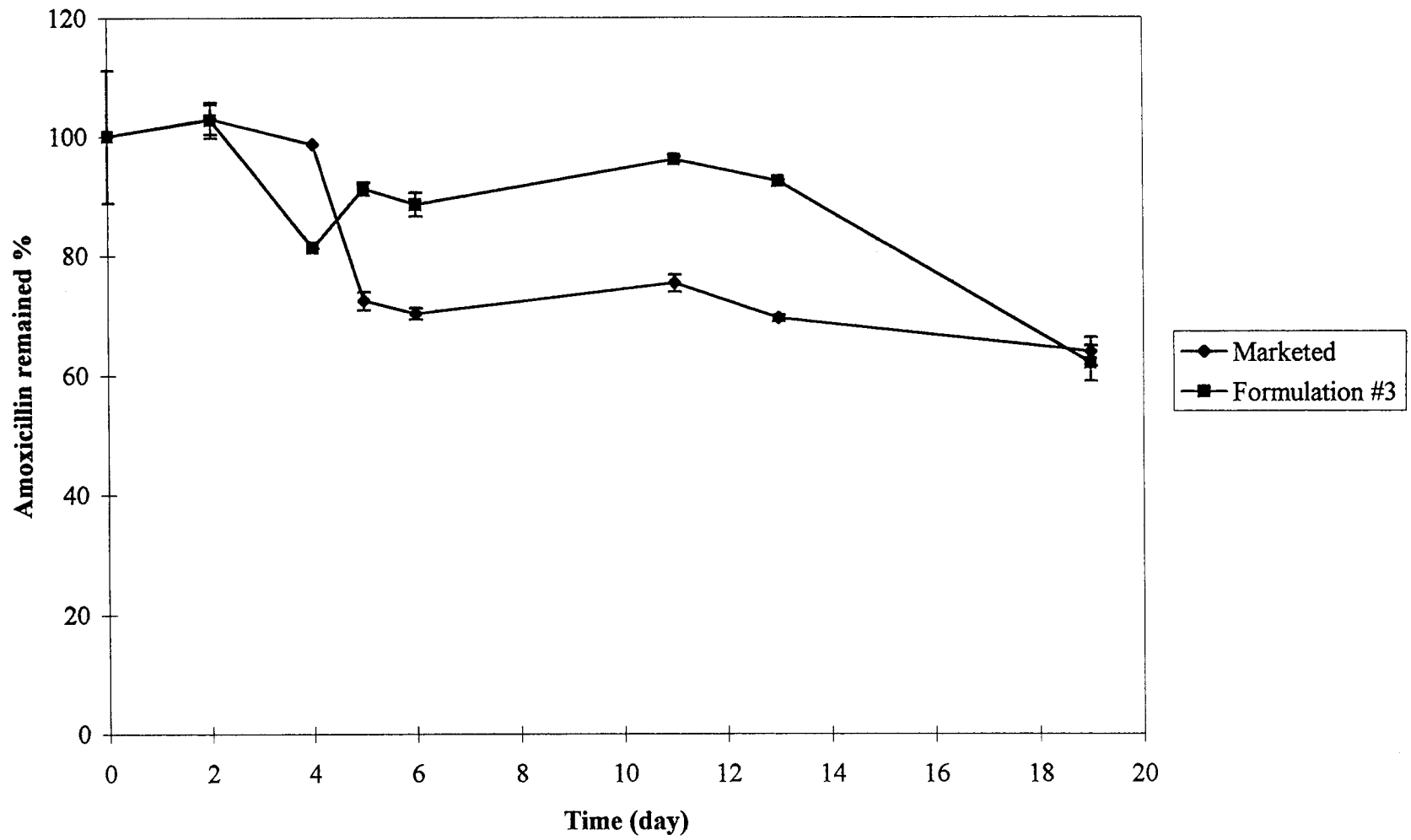


Figure 3.5. Amoxicillin stability at 40% relative humidity.

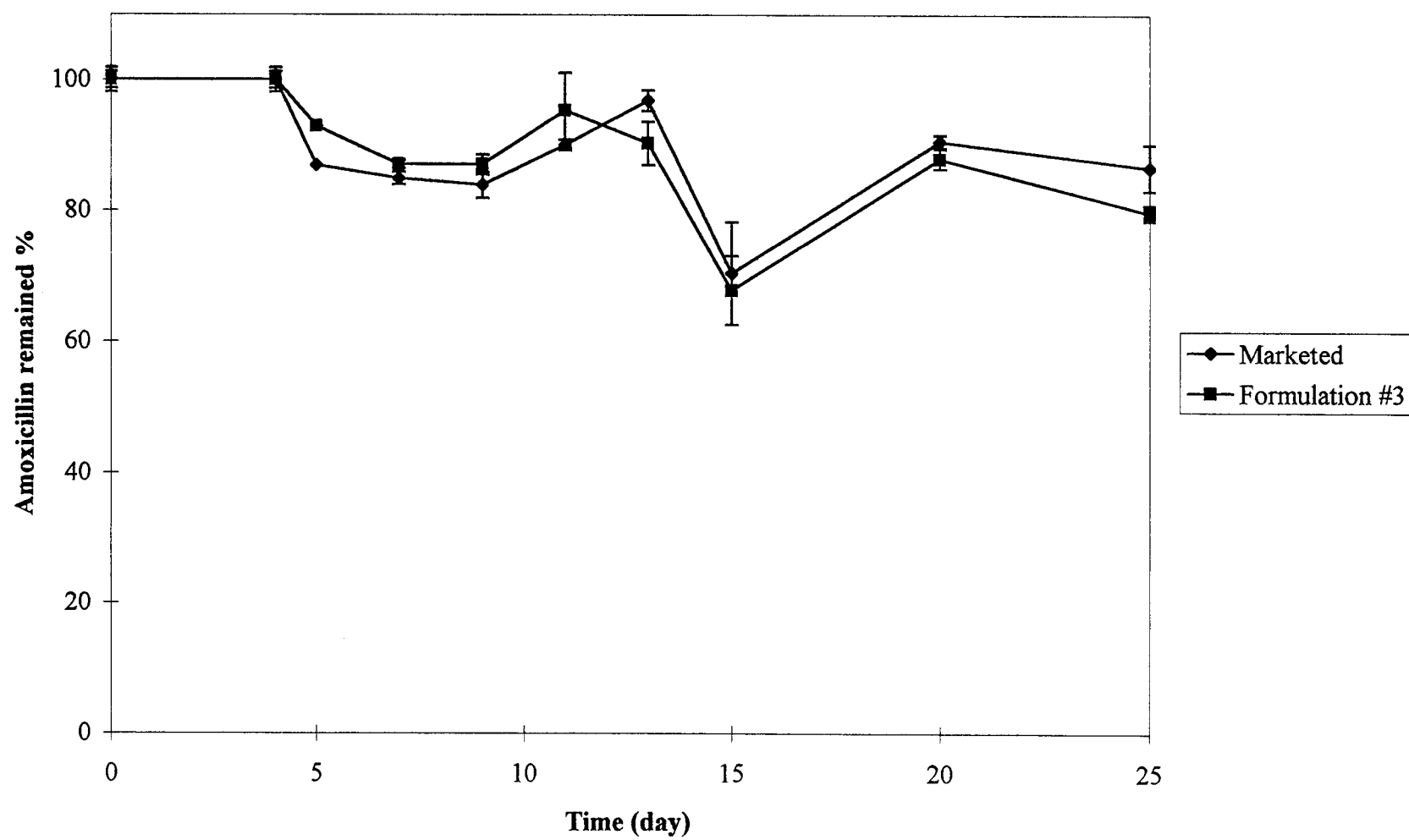


Figure 3.6. Clavulanic acid stability at 97% relative humidity.

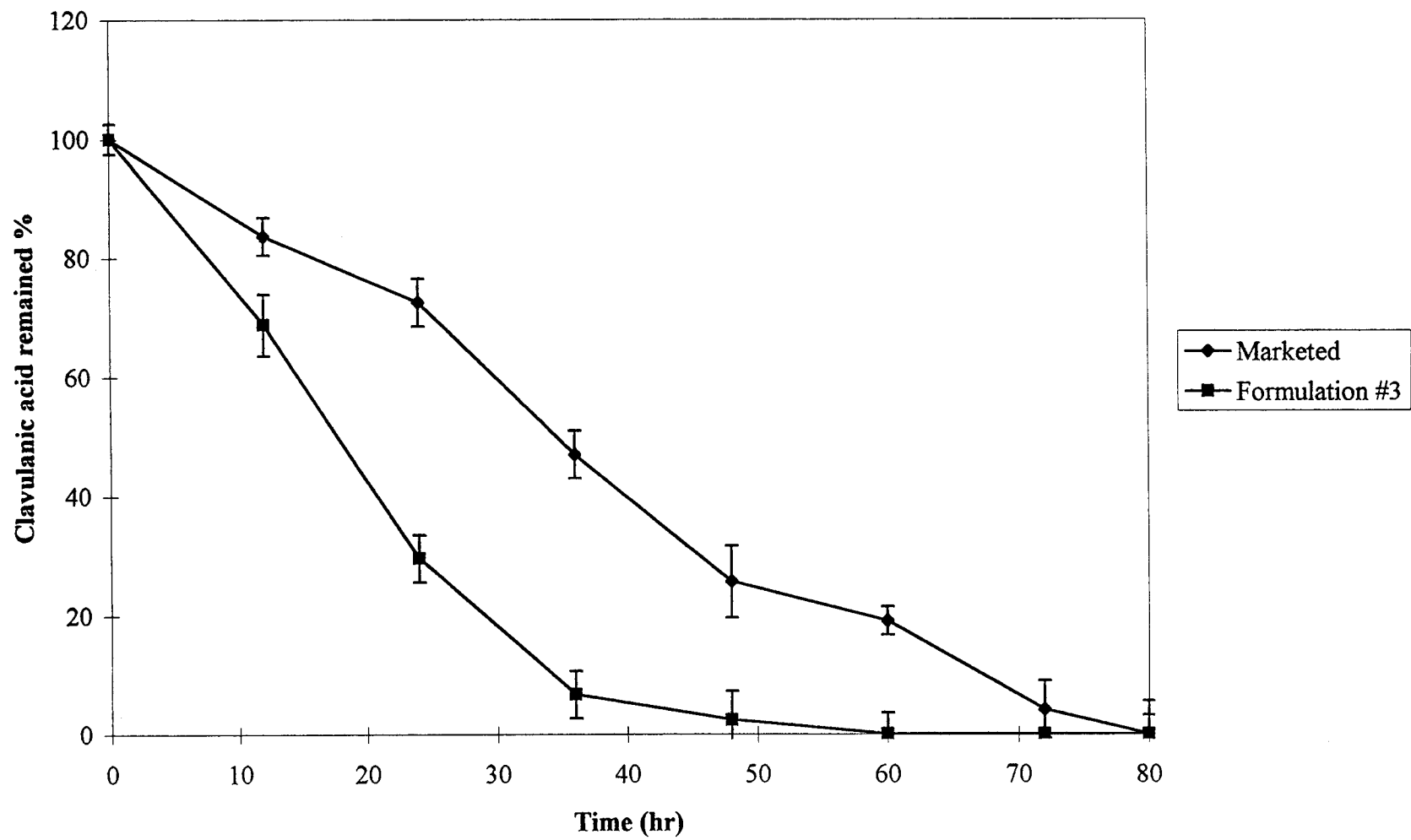
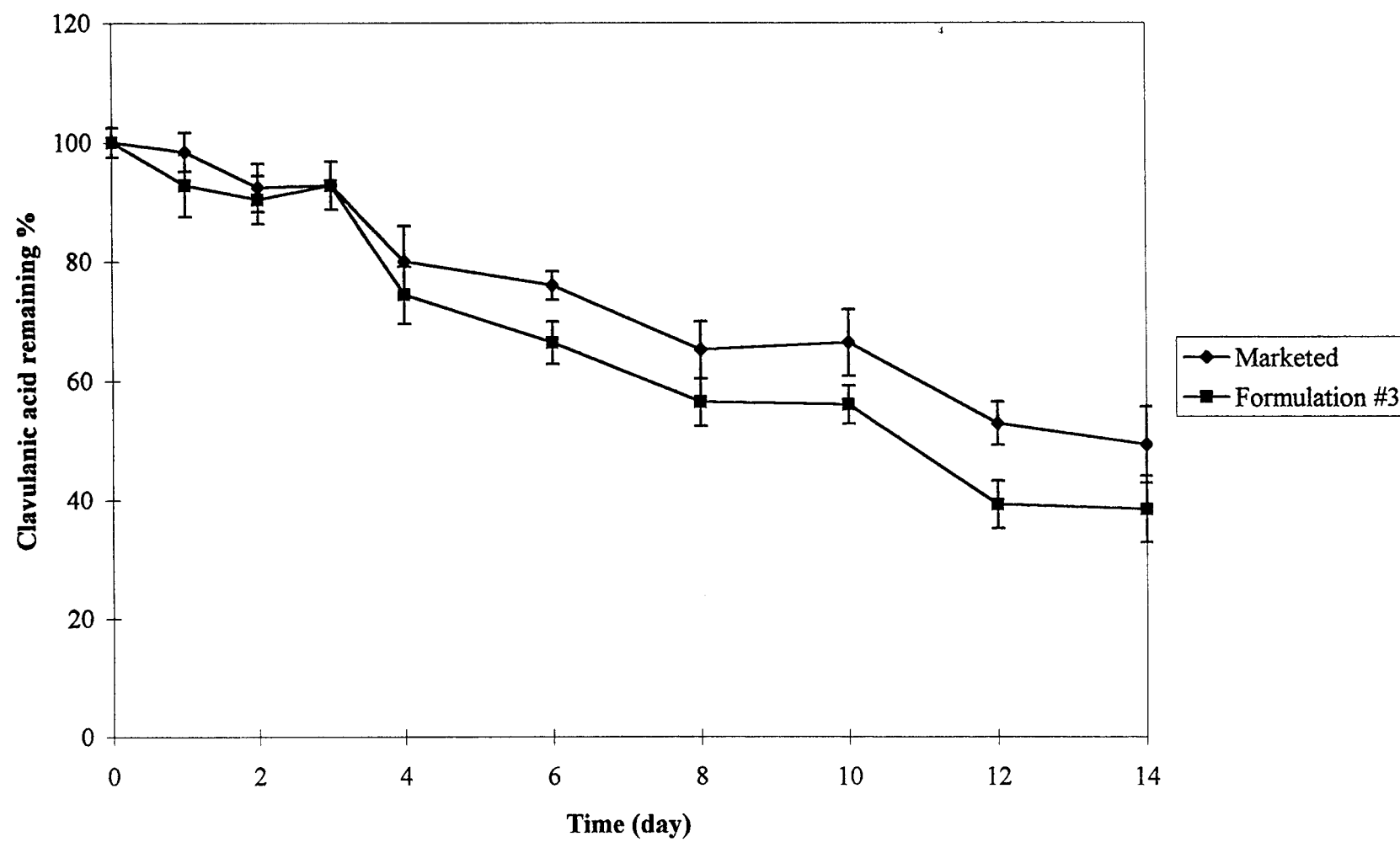


Figure 3.7. Clavulanic acid stability at 40% relative humidity.



Dissolution studies

Figure 3.8 shows that amoxicillin dissolves relatively faster from Formulation #3 than in the marketed formulation. This is due to the fact that the outer amoxicillin layer in the compressed-coated tablet disintegrates faster than a single layer mixture of amoxicillin and clavulanic acid.

Despite the difference in formulation design between the marketed formulation and Formulation #3, clavulanic acid in the two formulations showed similar dissolution profiles (Figure 3.9).

The outer layer, which did not bond very firmly to the inner core, might cap at the beginning of dissolution because of exposure to water, consequently, clavulanic acid dissolution may have started before complete disintegration of the outer coats.

Figure 3.8. Dissolution profiles of amoxicillin.

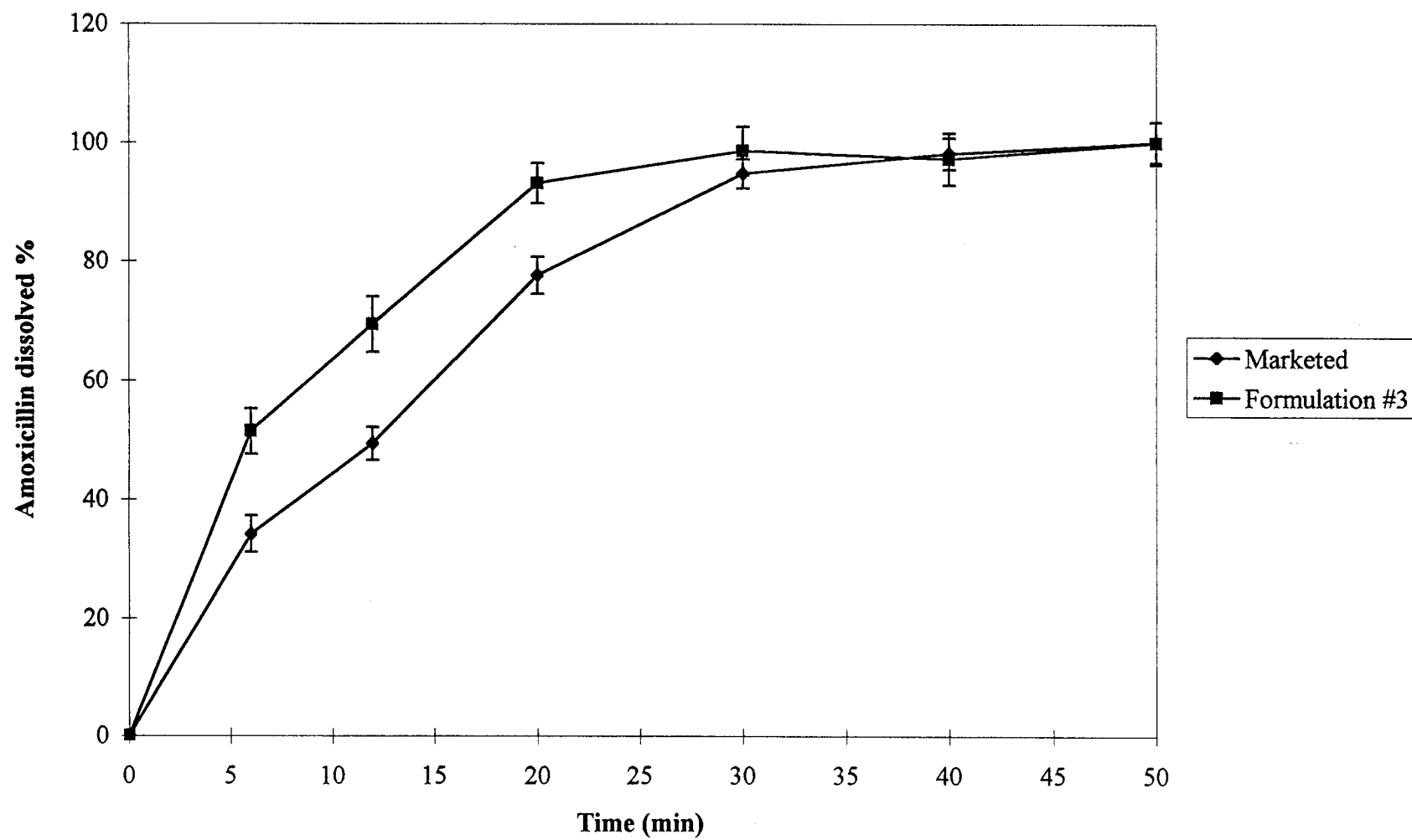
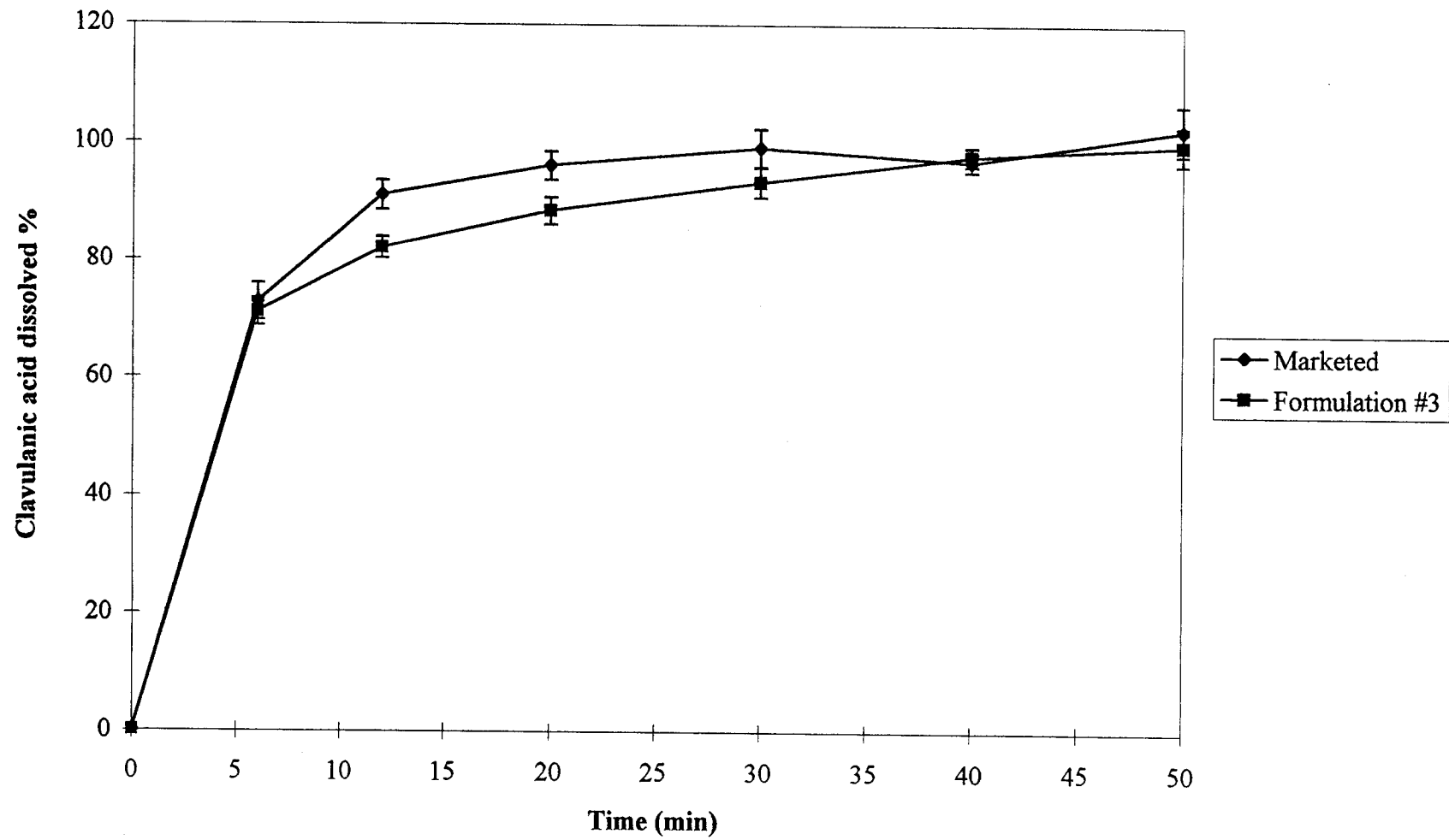


Figure 3.9. Dissolution profiles of clavulanic acid.



CONCLUSIONS

Compression coated tablets can be used to separate active ingredients in the same in dosage forms. It consists of three layers, and each layer must have good friability and compressibility. Consequently, compression of the outer coat into the hard inner core is challenging. The dosage for has been applied to amoxicillin and clavulanic acid. The outer layer containing amoxicillin showed cracks after compression into the inner coat containing clavulanic acid. The problem was minimized by incorporation of inactive ingredients that can have good friability at low compression force in the core, such as Emcompress[®]. Granulation of diluent in the outer layer with rapidly disintegrating acrylic polymers improved the bonding of the outer layer to the inner core. Cracking of the outer coat diminished its ability to form moisture barrier that will prevent the passage of moisture into the core, which lowered the stability of clavulanic acid.

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CHAPTER 4

BIOEQUIVALENCE OF AMOXICILLIN/CLAVULANATE COMPRESSED-COATED TABLET FORMULATION.

ABSTRACT

The bioequivalence of a new compression coated formulation, containing 500 mg amoxicillin as amoxicillin trihydrate, and 125 mg clavulanic acid as potassium clavulanate was compared to Augmentin[®]. Urinary excretion rates of both drugs were monitored as a non-invasive means to compare bioavailability. A randomized two-way crossover bioequivalence study was designed to evaluate bioavailabilities of both formulations in healthy human volunteers (5 men and 3 women) between the ages of 18 and 41. An average 76.65% and 13.96% of the administered dose of amoxicillin and clavulanic acid, respectively, were excreted unchanged in the urine within 6 hours after oral administration. Bioequivalence of the two formulations was evaluated using the power approach and the two one-sided t-test. According to the power approach, the two formulations were equivalent for amoxicillin and clavulanic acid. The results of the two one-sided t-test showed that AUC and C_{max} of amoxicillin for the tested formulation were within μ 20% of those of the reference product. However, AUC and C_{max} of clavulanate were not within μ 20%. Bioinequivalency of the two formulations for clavulanic acid might be due to small sample size and high intra-subject variation.

INTRODUCTION

Amoxicillin, D-(-)- α -amino-*p*-hydroxybenzyl-penicillin trihydrate, is an analog of ampicillin derived from the basic penicillin nucleus and has widespread therapeutic use. Clavulanic acid, Z-(3R, 5R)-2-(β -hydroxyethylidene) clavam-3-carboxylate is a potent inhibitor of β -lactamase enzymes, including those produced by *H. influenzae*, *S. aureus*, *N. gonorrhoeae*, and *Bacteroides fragilis* (1). Clavulanic acid has weak antibacterial activity itself (1). However, when combined with other β -lactam antibiotics like amoxicillin, the combination is very active against many bacteria resistant to the β -lactam alone. The usual adult oral dose of amoxicillin and clavulanate potassium is one 250 mg tablet containing 250 mg of amoxicillin and 125 mg of clavulanic acid every 8 hours (1). For more severe infections and infections of the respiratory tract, the usual adult oral dosage is one 500 mg tablet containing 500 mg of amoxicillin and 125 mg of clavulanic acid every eight hours (1).

Amoxicillin trihydrate and potassium clavulanate are both relatively stable in the presence of acidic gastric secretions and well absorbed following oral administration (1). Peak serum concentrations of amoxicillin and clavulanic acid are generally attained within 1-2.5 hours following oral administration of amoxicillin and potassium clavulanate in fasting adults (1). Serum concentrations of amoxicillin and clavulanic acid both decline in a biphasic manner and half-lives of the drugs are similar (1). Following oral administration of amoxicillin and potassium clavulanate in adults with normal renal function, amoxicillin has an elimination half-life of 1-1.3 hours and clavulanic acid has an elimination half-life of 0.78-1.2 hours (1). Following a single dose of amoxicillin and potassium clavulanate in adults with normal renal function, approximately 50-73% and

25-45% of amoxicillin and clavulanic acid, respectively, are excreted unchanged in the urine within 6-8 hours (1). Amoxicillin and clavulanic acid are both distributed into the lungs, pleural fluid, and peritoneal fluid. Low concentrations (i.e., less than 1 $\mu\text{g/ml}$) of each drug are attained in sputum and saliva (1). Only minimum concentrations of are attained in CSF following oral administration of amoxicillin and clavulanate potassium in patients with uninflamed meninges; higher concentrations may be attained when meninges are inflamed (1).

Aluminum hydroxide, milk and cimetidine have some influences on the bioavailability of a single dose of oral Augmentin, but the effects are unlikely to be of therapeutic importance (1). Oral administration of probencid shortly before or concomitantly with amoxicillin and clavulanate potassium slows the rate of renal tubular secretions of amoxicillin and produces higher and prolong serum concentrations of amoxicillin (3,4,5). However, probencid does not affect serum concentration of clavulanic acid, which might be due to the minor role of tubular secretion of clavulanic acid (5). Calcium channel blockade significantly enhances both absorption and bioavailability of amoxicillin without modifying its distribution or elimination (6); nifedipine can enhance intestinal amoxicillin intake by stimulating its active transport.

The objective of this study is to compare the bioavailability of amoxicillin and clavulanic acid from a compression-coated tablet (Figure 3.1) to that from the marketed conventional single layer tablet (Augmentin[®]). Each tablet of each formulation contains 500 mg amoxicillin as amoxicillin trihydrate and 125 mg clavulanic acid as potassium clavulanate. Hereafter, the compressed-coated formulation will be referred as the new formulation.

MATERIALS AND METHODS

Subjects

The study was approved by the Oregon State University Institutional Review Board for the Protection of Human Subjects. All subjects (3 female and 5 male) were healthy volunteers, between the ages of 18 and 41 years old (mean 25), and weighing between 130 and 190 lbs (mean 163). All subjects were non-smokers, with no known allergic reaction to penicillin or any other antibiotics, and were not on any previous or current medication including. None of the subjects had history of gastrointestinal, kidney, or liver disease.

Study design

After overnight fasting the day of the study, the subjects had a standard breakfast which consisted of a plain bagel, one ounce of cream cheese and a 236 ml juice drink (Sunny delight®). Absorption of amoxicillin and clavulanic acid from Augmentin® is reported to be unaffected by food. Therefore, Augmentin® may be administered with meals which can minimize the possibility of GI tract disturbances. Either the compressed-coated tablet formulation or Augmentin® was assigned as a starting dose and the tablet was given within 5 minutes after breakfast was taken. Subjects were instructed not to eat or drink tea or coffee for the next two hours with no subsequent restrictions. Urine was collected in labeled Whirl-Pak® bags at 1, 2, 3, 4, 5 and 6 hours after administration for HPLC analysis. After at least a 24 hour washout period, the alternate product was given to subjects.

Liquid chromatography

Amoxicillin and clavulanic acid were analyzed separately using two independent HPLC systems. A Waters solvent pump (Waters Associates, Inc., Milford, MA, U.S.A.) was used to pump degassed mobile phase through a C18 reverse-phase column (inner diameter 4.6 mm by 25 cm, particle size, 5 μ m; Rainin Instrument Company, Inc., Woburn, MA) with a flow rate of 1.3 ml/min for amoxicillin and 2.0 ml/min for clavulanic acid. The eluent was monitored for amoxicillin at 229 nm using Waters model 441 absorbance detector. In order to lengthen the retention time and obtain distinct peaks from interfering components in urine (7), clavulanic acid was assayed by reacting the sample with imidazol; the derivative was detected at 313 nm using Waters model 440 absorbance detector. The mobile phase was 0.0005M potassium dihydrogen orthophosphate in 5% methanol in deionized water for amoxicillin and 0.001M potassium dihydrogen orthophosphate in 6% methanol in deionized water (adjusted to pH 3.0 with phosphoric acid) for clavulanic acid. Injection volumes were 20 μ l and 50 μ l for amoxicillin and clavulanic acid, respectively. Absorbance of amoxicillin and clavulanic acid was recorded individually through two channels of a Shimadzu CR501 Chromatopac (Shimadzu Corporation, Chromatographic & Spectrophotometric Instruments Division, Kyoto, Japan).

Sample preparation for HPLC analysis

Once the urine sample was returned to the laboratory, the full volume was measured and 1 ml urine was immediately buffered to pH 6 to ensure stability. Sulfadiazine and paracetamol, at concentrations to achieve appropriate peak area ratio, were used as internal standards for amoxicillin and clavulanic acid, respectively. The imidazole reagent for clavulanic acid derivitization was prepared by dissolving 8.25 gm of imidazol in 24ml deionized water plus 2 ml of 5M HCL. The solution was adjusted to pH 6.8 by addition of 5M HCL, and volume was made up to 40 ml with deionized water. Sample for clavulanic acid analysis was prepared by reacting 100 μ l solution containing clavulanic acid with 400 μ l imidazol reagent for 10 minutes, followed by the addition of 50 μ l sulfadiazine. Samples for amoxicillin analysis were mixed with equal volumes of paracetamol before HPLC analysis.

Data analysis

WIN-NONLIN[®] was used to calculate bioavailability parameters (C_{max} , T_{max} and AUC_{0-4}). C_{max} is defined as the maximum urinary excretion rate of drug and T_{max} is the time corresponding to the maximum urinary excretion rate of drug. AUC is defined as the area under the curve depicting excretion rate plotted with respect to midpoint of time interval. C_{max} and T_{max} were directly taken from the data. AUC was estimated by the trapezoidal rule. AUC and C_{max} were statistically analyzed by ANOVA using SAS[®] and T_{max} was analyzed by the non-parametric statistical test Wilcoxon rank-sum test using S-Plus[®].

RESULTS AND DISCUSSION

Figures 4.1 and 4.2 show that the mean excretion rates for the two formulations for amoxicillin and clavulanic acid are similar. The pharmacokinetic parameters of both drugs in each formulation are shown in Tables 4.1 and 4.2. Previous bioequivalence study of two compression-coated chewable tablet formulations and the marketed chewable tablet (each contained 250 mg amoxicillin and 62.5 mg clavulanic acid) (8) showed that the average C_{max} and AUC in the three formulations were 62.19 and 174.14, respectively, for amoxicillin and 4.33 and 9.96, respectively, for clavulanic acid. Compared to these results, The average values of AUC and C_{max} in Tables 4.1 and 4.2 are approximately doubled for the doubled dose (500 mg), which would suggest that both amoxicillin and clavulanic acid follow linear pharmacokinetics.

Compared to 76.87% and 14.61% for the marketed formulation, it was calculate that 76.43% and 13.30% of amoxicillin and clavulanic acid, respectively, were excreted unchanged within 6 hours of administration of the compression-coated tablet formulation.

Power approach and two one-sided test procedures (9,10) were used to assist in evaluating bioequivalence of the two formulations.

In the power approach, a decision on the bioequivalence of two formulations is based solely upon a test of the null hypothesis (H₀):

$$H_0: \mu_T - \mu_R = 0$$

Against the alternative hypothesis (H₁):

$$H_1: \mu_T - \mu_R \neq 0$$

where μ_T is the population mean for the test formulation and μ_R is the population mean for the reference formulation.

Figure 4.1. Mean urinary excretion rates of amoxicillin.

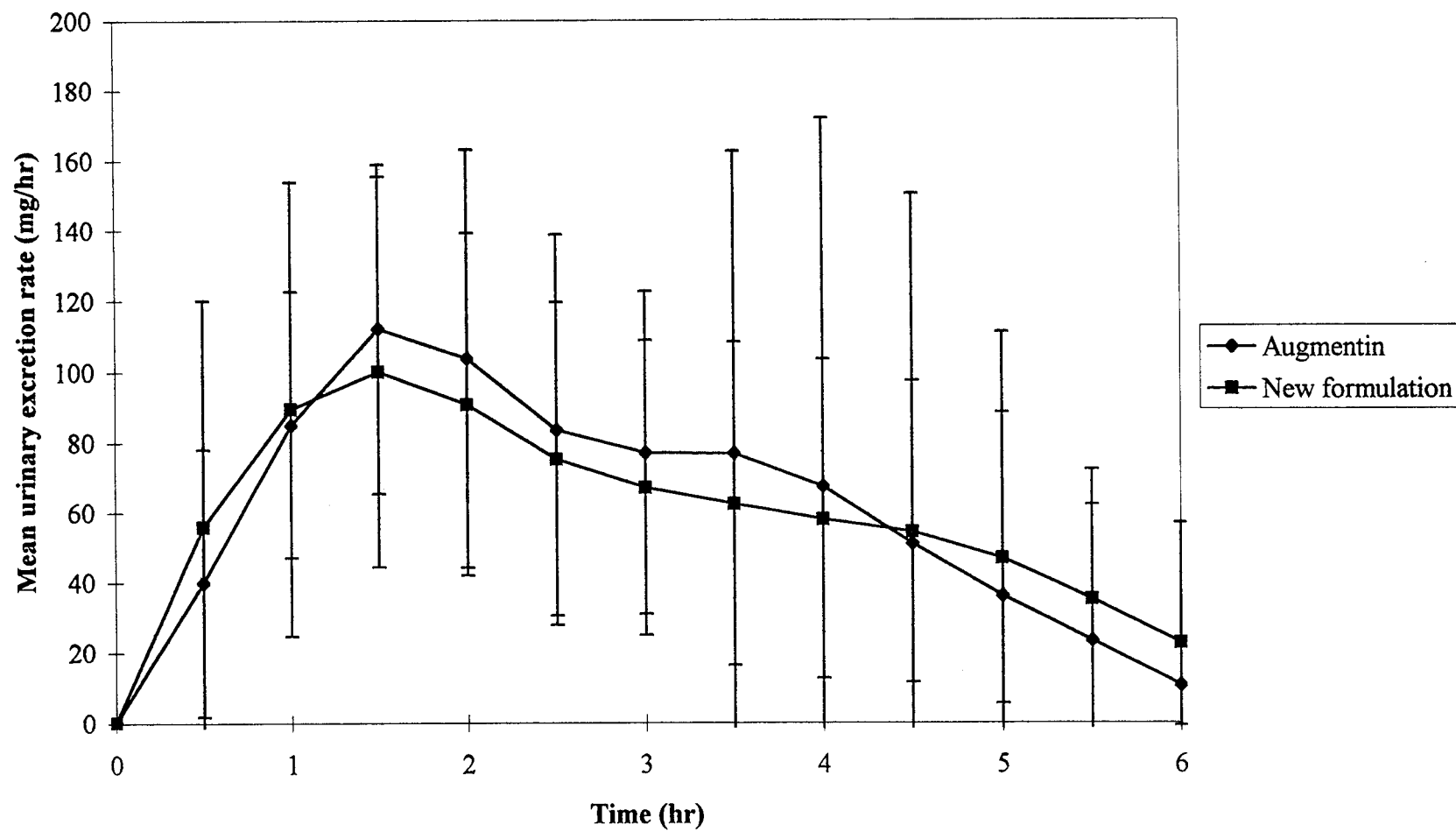


Figure 4.2. Mean urinary excretion rates of clavulanic acid.

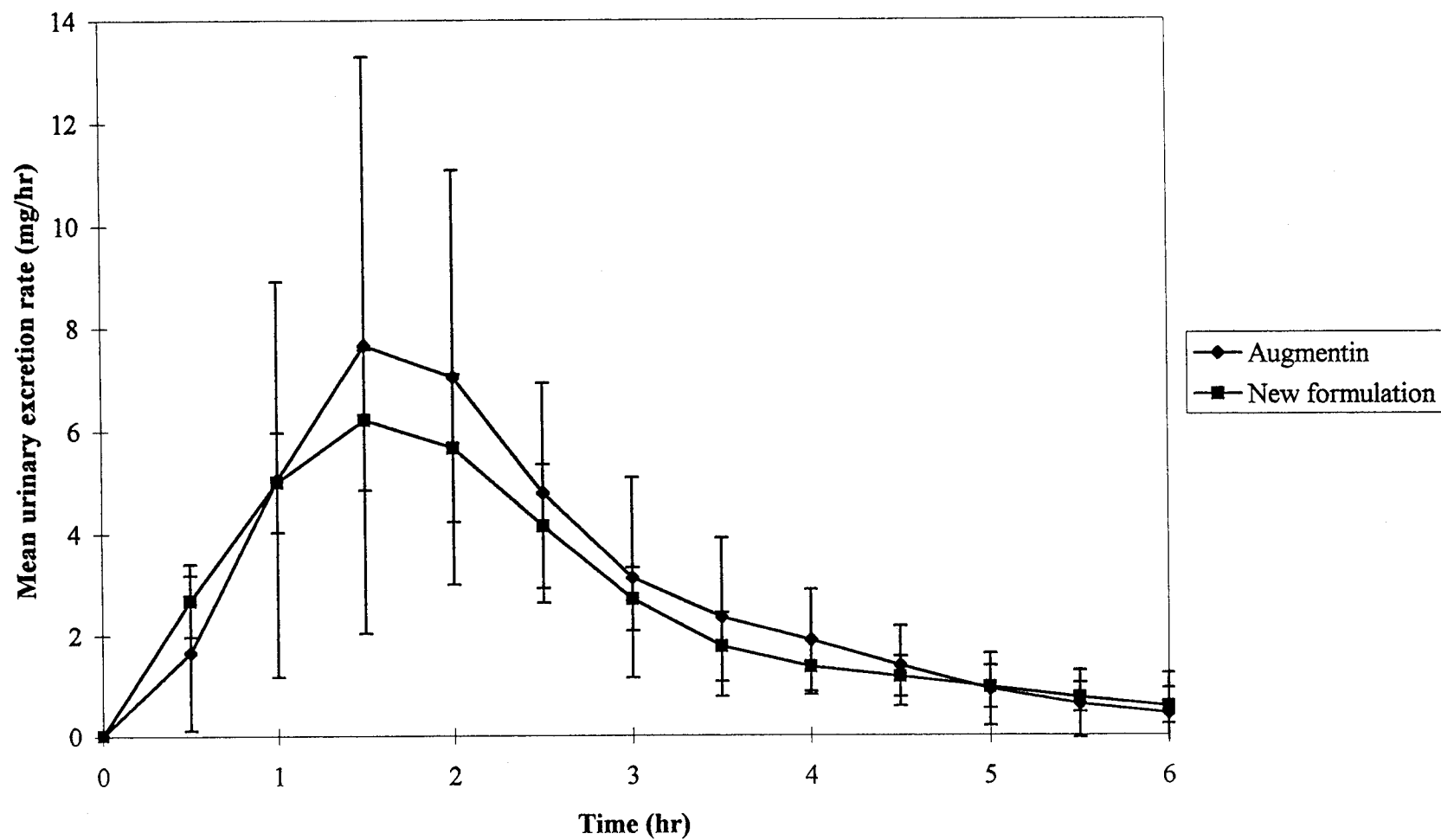


Table 4.1. Amoxicillin pharmacokinetic parameters.

Subject	Formulation					
	Augmentin [®] New		Augmentin [®] New		Augmentin [®] New	
	Cmax		Tmax		AUC _{0-t}	
1	170.85	191.76	1.50	1.5	430.92	445.99
2	161.36	126.35	2.50	1.5	371.15	379.87
3	130.66	141.22	1.50	3.59	292.87	578.90
4	54.00	66.24	1.50	3.42	135.48	199.22
5	116.59	179.38	1.45	0.44	347.46	272.81
6	131.40	57.27	1.50	4.72	189.46	104.48
7	296.02	135.98	4.50	2.5	727.70	505.88
8	157.59	110.87	2.50	2.5	418.23	332.12
Average	152.31	126.13	2.12	2.52	364.16	352.41
SD	68.61	47.85	1.07	1.37	180.38	158.59
CV	45.05	37.94	50.27	54.47	49.53	45.00

Table 4.2. Clavulanic acid pharmacokinetic parameters.

Subject	Formulation					
	Augmentin [®] New		Augmentin [®] New		Augmentin [®] New	
	Cmax		Tmax		AUC _{0-t}	
1	14.90	9.56	1.50	1.50	28.10	25.56
2	5.05	5.91	2.50	1.50	13.36	11.30
3	6.29	6.29	2.50	3.60	16.89	16.14
4	1.75	4.05	1.50	1.50	3.43	7.23
5	6.93	5.32	1.45	0.44	19.34	10.27
6	15.60	1.12	1.5	2.80	28.98	4.23
7	8.40	12.03	1.5	1.50	14.82	26.50
8	9.66	9.43	1.5	1.50	16.19	23.67
Average	8.57	6.71	1.74	1.79	17.64	15.61
SD	1.68	3.48	0.47	0.97	8.21	8.70
CV	55.33	51.87	26.79	53.92	46.54	55.72

Analysis of variance (ANOVA) is the standard method of choice for evaluating a bioequivalence study using the power approach. Using an F-ratio, the null hypothesis of no difference between the formulations is tested at the 5% level of significance. Differences between subjects and between periods can be tested at the same time. Significant differences between subjects will almost always be present and reflects biological variation between individuals. Significant differences between periods are not expected to be found.

For the parameter of T_{max} , the use of ANOVA is not appropriate. The values obtained for this parameter come from a discrete data set (i.e. the preselected sampling times) and can be poor estimates of the true value. It is recommended that a nonparametric method, such as Willcoxon statistic, be used to test for differences in T_{max} between the formulations.

The two one-sided tests procedure involves testing two interval hypotheses

$$H_{01}: \mu_T - \mu_R < \theta_1 \quad (\text{right tail})$$

$$H_{11}: \mu_T - \mu_R \geq \theta_1$$

and

$$H_{02}: \mu_T - \mu_R > \theta_2 \quad (\text{left tail})$$

$$H_{12}: \mu_T - \mu_R \leq \theta_2$$

The two one-sided tests procedure consists of rejecting the interval hypothesis H_0 and thus concluding equivalence of μ_T and μ_R , if, and only if, both H_{01} and H_{02} are

rejected at a chosen nominal level of significance. The logic behind this is that if one may conclude that $\theta_1 < \mu_T - \mu_R$, and also conclude that $\mu_T - \mu_R < \theta_2$, then it has in effect been concluded that $\theta_1 < \mu_T - \mu_R < \theta_2$. Under the normality assumption that has been made, the two sets of one-sided hypotheses will be tested with an ordinary one-sided t-test. For a balanced study, it will be concluded that μ_T and μ_R are equivalent if

$$t_1 = \frac{(\bar{X}_T - \bar{X}_R) - \theta_1}{S \sqrt{\frac{1}{n_T} + \frac{1}{n_R}}} \geq t_{1-\alpha}(\nu) \quad t_2 = \frac{\theta_2 - (\bar{X}_T - \bar{X}_R)}{S \sqrt{\frac{1}{n_T} + \frac{1}{n_R}}} \geq t_{1-\alpha}(\nu)$$

where S is the square root of the “error” mean square from the crossover design analysis of variance. $t_{1-\alpha}(\nu)$ is the point that isolates probability α in the upper tail of the Student’s t distribution with ν degrees of freedom, where ν is the number of degrees of freedom associated with the “error” mean square.

Both the power approach and two one-sided tests are based on the assumptions of normal distribution and constant variance (10). A logarithmic transformation has the effect of bringing the distribution of data closer to a normal distribution and stabilizing the variance. It is often instructive to analyze the data twice, once with, and once without, a logarithmic transformation. In the large majority of cases, transforming the data has little or no influence on the final statistical outcome. It is now recommended that C_{max} and AUC data should be logarithmically transformed prior to statistical analysis (10). In these cases the acceptance region for bioequivalence should range from 80% to 125%.

Table 4.3 shows that according to the power approach the two formulations are equivalent for C_{max} and AUC for both amoxicillin and clavulanic acid. Also, the two

formulations are equivalent for Tmax according to Wilcoxon sum-rank test. Based on two one-sided t-test (Table 4.4), the Cmax and AUC of the tested formulation are within μ 20% of those of the reference product for amoxicillin but not for clavulanic acid.

Table 4.3. P-values for differences between formulations obtained by ANOVA for Ln-Cmax and Ln-AUC_{0-t} and by Wilcoxon rank-sum test for Tmax.

Amoxicillin				Clavulanic acid		
Parameter	Ln-AUC _{0-t}	Ln-Cmax	Ln-Tmax	Ln-AUC _{0-t}	Ln-Cmax	Ln-Tmax
p-Value	0.80	0.33	0.42	0.64	0.52	0.90

Table 4.4. t-Statistics calculated according to two one-sided t-test.

	Parameter	Augmentin®	New	MSE*	t_1	t_2
		formulation				
Amoxicillin	Ln-AUC _{0-t}	5.786	5.748	0.086	17.22	-24.29
	Ln-Cmax	4.934	4.762	0.106	3.86	-29.82
Clavulanic acid	Ln-AUC _{0-t}	2.725	2.58	0.358	1.75	-8.23
	Ln-Cmax	1.975	1.724	0.54	-0.41	-7.02

*Mean square error obtained from ANOVA table.

The bioinequivalency of the two formulations for clavulanic acid might be due to the high intra-individual variability and small sample size. When the variation is large because of the inherent biological variability in the absorption and/or disposition of the drug (or due to the nature of the formulation), large sample sizes may be needed to meet the confidence interval criterion (11). Also, when the intra-subject variability is high the two one-sided t-test may not be appropriate, and indeed widened acceptance limits (70-143%) may be recommended for bioequivalence testing (12).

To calculate the optimum sample size needed to detect bioequivalency of the formulations, for both drugs (for AUC and C_{max}), the following power calculation was used (11)

$$N = (\sigma / \Delta)^2 (Z_{\alpha} + Z_{\beta})^2 + 0.5(Z_{\alpha 2}) \quad \text{Eq. [1],}$$

where N = sample size, α = level of significance (5%), β = 1-Power (0.2), σ = standard deviation of the ratios of test:reference pharmacokinetic parameters, Δ = a “practically significant” difference (0.2). Z values are obtained from statistical tables.

According to Table 4.5, AUC and C_{max} of clavulanic acid are the most variable, consequently, the optimum sample size to test for bioequivalence of the two formulations is 85 which is about 10 fold of the sample size that was used in this study. However, AUC for clavulanic acid for the new formulation was 88% of clavulanic acid AUC for the reference, and C_{max} was 78%. It seems clear that AUC is bioequivalent between the two formulations without conducting a study in 85 people. C_{max}, however, can not be considered bioequivalent at this time.

Table 4.5. Optimum sample size calculated according to Eq. 1.

	σ	N
Amoxicillin		
AUC _{0-t}	0.42	37
C _{max}	0.39	32
Clavulanic acid		
AUC _{0-t}	0.65	85
C _{max}	0.65	85

CONCLUSIONS

Amoxicillin and clavulanic acid are excreted in measurable amounts in urine, which makes urine excretion data useful in measuring their pharmacokinetic parameters. A new compression coated tablet formulation of amoxicillin and clavulanic was evaluated for bioequivalency to the marketed conventional single layer tablet formulation. Both formulations were bioequivalent for amoxicillin for all the required pharmacokinetic parameters. However the two formulation were not bioequivalent for clavulanic acid for AUC and C-max. Sampling size calculations, based on the pharmacokinetic parameters of the two active ingredients, showed that the number of subjects required to reveal any inequivalency between the two formulations is about three times the number of subjects participated in the study.

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CHAPTER 5

CHARACTERISTICS OF NISIN

STRUCTURE

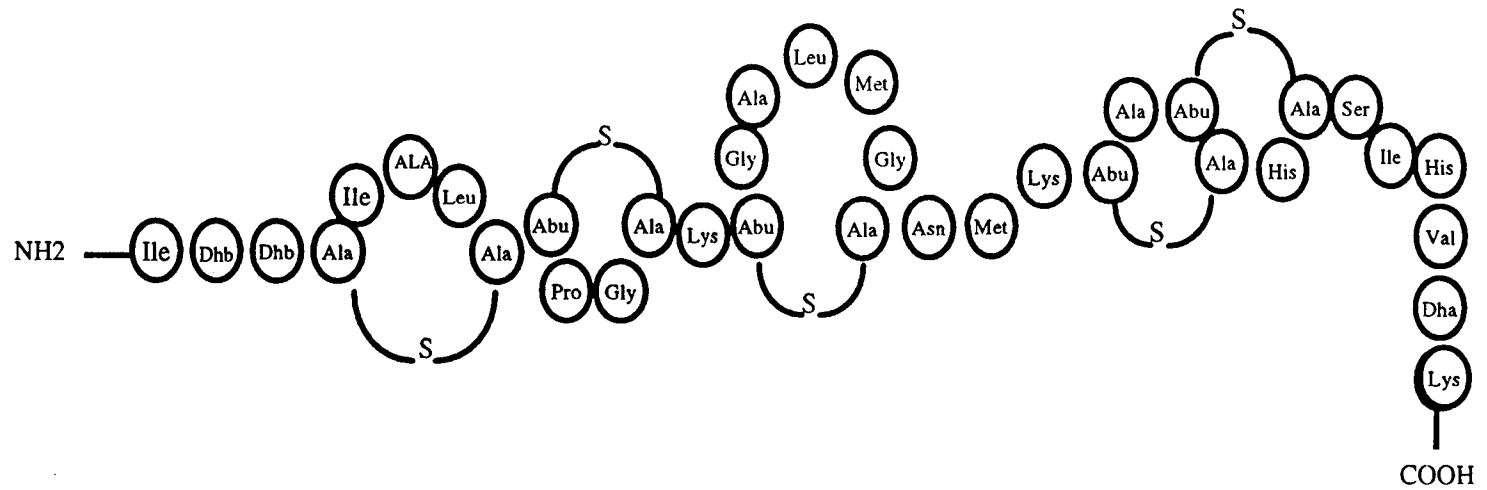
Nisin consists of 34 amino acid residues. The molecule possesses amino and carboxyl end groups, and five thioether bands form internal rings (1) (Figure 5.1). Although molecular mass of the nisin monomer is about 3500 (2), nisin usually occurs as stable dimers of a molecular mass of 7000 or tetramers of a molecular mass of 14000 (3, 4).

PHYSICAL AND CHEMICAL PROPERTIES

As far as the amino acid is concerned, nisin possesses an amphiphilic character, with a cluster of bulky hydrophobic residues at the N-terminus and hydrophilic ones at the C-terminus (5). The distribution of polar and apolar residues over the molecular surface of nisin may be of relevance with respect to its mode of action and biological activity. Furthermore, nisin behaves as a cationic polypeptide with a positive charge of 3 and hence an isoelectric point in the alkaline range. Nisin contains no aromatic amino acids, so it does not absorb UV-light at 260 or 280 nm (6).

The solubility of nisin is highly dependent on the pH of the solution. It drops sharply and continuously as the pH is increased. In neutral and alkaline conditions, nisin is almost insoluble (7-10). The solubility ranges from 57 mg/ml at pH of 2.0 to about 1.5 mg/ml at pH of 6.0; it drops further to 0.25 mg/ml at pH of 8.5, and then it levels off (9). In addition, nisin solubility is inversely related to buffer concentration (9). In non aqueous solvents, nisin is almost completely insoluble (11)

Figure 5.1. Structure of nisin. Abu: 2-aminobutyric acid; Dha: dehydroalanine; Dhb: dehydrobutyrine.



The stability of nisin is also pH dependent, with maximum stability occurring at near pH 2 (9) and significant inactivation occurring at alkaline pHs greater than pH 7 (10,12). Indeed, nisin solution can be boiled in dilute hydrochloric acid at pH 2.5 or less without any loss of activity. Moreover, nisin remains stable after autoclaving at 115.6 °C at pH 2.0 but losses 40% of its activity at pH 5.0 and more than 90% at pH 6.8 (13). The stability of nisin solution to heating and storage depends not only on pH, but also on several other factors such as the chemical composition of the solution, the protective effect of proteins, the temperature, etc. For instance, nisin can be irreversibly adsorbed by some proteins. This adsorption probably accounts for the protective effect when nisin solutions are exposed to heat (8).

BIOLOGICAL PROPERTIES

Inhibition spectrum

Nisin displays activity against Gram-positive bacteria, and has proven effective against a variety of spoilage bacteria in foods (11,14). Although normally not active against Gram-negative bacteria and fungi, nisin can be an effective inhibitor of certain Gram-negative bacteria when used in combination with other compounds such as chelating agents (15,16). In high concentrations, nisin has also shown activity against yeast (17). Of special interest, however, is nisin activity against several hazardous food pathogens, including *Listeria monocytogenes* (18,19), and sporeformers such as *Clostridium botulinum* types A, B, and E (20).

Mechanism of nisin activity

The exact mechanism of nisin antimicrobial activity is not completely understood, but it is generally believed that nisin acts on the bacterial cytoplasmic membrane, leading to pore formation, loss of cellular integrity, and subsequent cell death (14,21,22). The first step in the antibacterial process involves adsorption of nisin to susceptible bacteria (8). Normally nisin does not adsorb to Gram-negative bacteria, likely due to the barrier function of the lipopolysaccharide membrane characteristics of most Gram-negative species (23,24). However, following sub-lethal injury, many Gram-negative bacteria became sensitive to nisin (16). This was also observed for nisin producer strains such as *Lactococcus lactis* ATCC 11454 and *Lc lactis* 345/07, which are normally resistant to bacteriocin but became sensitive following freezing (25). Once adsorption has taken place, a change in nisin conformation is believed to occur, and the hydrophobic moieties of the peptide are drawn into the hydrophobic bacterial membrane (26). Binding should also lead to increasing concentrations of the peptides at the membrane as compared to the concentration in solution (24), which in turn increases the probability of oligomerization and formation of peptide preaggregates (24). Due to the amphiphilic nature of nisin, charges are exposed on one side of the molecule which makes it rather unlikely that a peptide monomer could adopt a transmembrane orientation; moreover, a monomer should not be able to form a channel of the size determined in planar membrane experiments (24). Thus, oligomerization seems to be essential and should occur at the membrane surface (24). Increasing the energy of the membrane should then force the peptide oligomer into a conducting state, presumably by adopting a transmembrane orientation in

such a way that the hydrophobic face of the aggregate is exposed to surrounding lipids while the hydrophilic face forms the inner lumen of the pore (24).

THE USE OF NISIN

Toxicity of nisin

The fact that nisin is produced by *Lactococci* which occur naturally in raw milk and cheese is an indication of its harmless nature: it has been ingested by humans and animals over past centuries, without apparent ill effect. While this does not rule out the possibility that nisin might have some adverse effects, it does indicate, at least, that nisin has low toxicity (27). The LD50 value of nisin was found to be similar to that of common salts, i.e. about 7g/kg body weight (28). Furthermore, consumption of nisin containing products would not result in alteration of the intestinal bacterial flora, because nisin is inactivated by the enzymes of the intestinal tract (29,30). Moreover, nisin has no action on Gram-negative bacteria, which form substantial part of the intestinal flora (31). In addition, nisin cannot be detected in the saliva of humans 10 min after consumption of chocolate milk containing 200 IU/ml nisin and hence would not be expected to alter the nature of bacterial flora in the oral cavity (32). Furthermore, there is no evidence of sensitization to nisin in human beings (31).

Applications

Nisin is mainly used in the production of food to prevent spoilage by Gram-positive bacteria, especially *Clostridium*, *Staphylococcus*, *Bacillus* and *Listeria* (12). In 1989, nisin had been approved for use in many countries, under different restrictions (1). In 1988, the food and drug administration has affirmed the “generally recognized as safe” (GRAS) status given to nisin used in the production of certain pasteurized processed spread cheese (33,34). The addition of nisin to meat allows for a reduction of the nitrite content in the food (10). Nisin has been shown to delay the production of toxin by *Clostridium botulinum* in fresh fish (35). In the production of beer, the addition of nisin may help in preventing spoilage due to lactic acid bacteria (36-38). The addition of nisin to canned vegetables and fruits has been demonstrated to allow milder heat treatment (39). In cosmetics, nisin is used to increase the shelf life of certain products, e. g., cosmetic ointments (12).

PROTEIN-STABILIZED EMULSIONS

Proteins are by far the most important class of emulsifiers of oil-in-water emulsions in foods. They are amphiphilic molecules, and tend to bind to hydrophobic interfaces via hydrophobic parts of their structures. Stabilization of oil droplets in water is achieved by protein adsorption to the droplet surface as it can impart an electronic charge to droplet surfaces (40,41), result in steric stabilization (42), or both. Approaching particles would experience an energy barrier higher than thermal energy and so not approach closely enough to interact (43).

Practical measurements of emulsifying activity of proteins generally results from combination of several factors, and it is often difficult to relate the molecular adsorption to the overall emulsifying properties because the structure, charge, composition and strength of the interfacial layer are all important. The amounts of individual proteins which adsorb to an oil-water interface do not seem to differ widely, and there is at present no single property of a protein which will allow prediction of how it will adsorb in the presence of other surface-active components. The functionality of an emulsion droplet is determined partly by the conformation which is adopted by the adsorbed protein, and this in turn depends on the primary, secondary and tertiary structures, the strengths of the bonds which maintain the secondary structure, and the conformational flexibility of the protein, the spatial arrangement of hydrophobic areas on the surface of the molecule, and the potential for intermolecular bond formation between adsorbed molecules.

In general, emulsions are stable if the oil-water interface is surrounded with thick adsorbed layer of protein or other molecules which resists breakdown and thinning, so that the oil droplets are prevented from coalescing. To achieve this, the adsorbed molecules must lower the interfacial tension by as large an amount as possible, and this is most readily achieved when they possess considerable hydrophobic character. The ability of a protein to cover an interface is critical during the early stages of emulsion formation. All proteins make a layer of some thickness on the oil-water interface, and therefore may present a more rigid interfacial layer than do small molecule emulsifiers. In terms of stability, the proteins may be more effective, but in terms of overall effectiveness, it is often the smaller molecules which play an important part. The interplay between these two types of emulsifiers forms a new and important area for research.

NISIN AT INTERFACES

Nisin is a highly surface active molecule whose adsorptive properties were recognized as early as 1949 when it was noted that nisin in culture remained with *Lactococcus lactis* cells rather than in the culture broth at pH 6 (44). In 1951, Friedman and Epstein (45) recognized that nisin could adsorb onto glass test tubes, and that 2 U/ml could be released when sterile, fresh broth was added to the empty tubes. They also found that even boiling with detergents and oxidizing agents was not capable of removing all the adsorbed nisin. Nisin adsorption at silanized silica and the retention of its antibacterial activity after adsorption was studied. It was found that nisin can adsorb to solid surfaces in a manner stable to rinsing, maintain activity and kills bacteria cells that have adhered (46-48). A patent on preparation of antibacterial surfaces with adsorbed bacteriocin was recently issued (49). It was shown that the adsorption of nisin air-water interface results in considerable reduction in surface tension (50). The adsorption of nisin at bacterial cell is pH dependent (51), with maximum adsorption at pH 6.5, and less than half adsorbed at pH 4.4 (11). Nisin has been considered to produce the same outcome as cationic, surface-active detergent effect on the bacterial cell membrane because leakage of UV absorbing material from treated bacterial cells was observed (52). The adsorption of nisin to glass, bacterial cells, and the air-water interface does not, however, suggest the same mechanism of action as an emulsifying agent. Quite the opposite is expected. Nisin's adsorption at surfaces may be related to its' low water solubility. Nisin is also almost completely insoluble in nonaqueous solvents. Emulsifying surfactants must be attracted to both water and oil and orient into micelles with either water entrapped in oil or oil entrapped in water as a result of the emulsifier solubility in either water or oil. Thus,

although nisin has adsorptive properties at cell and glass surfaces, it should not solubilize in oil-in-water mixture and not dissolve cell membrane by dissolving their structure into oil-in-water micelles. In fact, it is known that nisin does not work through such emulsifying action but rather works by adsorbing to bacterial cell walls and forming pores and channels through biological membranes.

Caseins are probably the most widely used of the food proteins which have known emulsifying activity. They possess a relatively small amount of regularly tertiary structure and are conformationally mobile. They possess extensive hydrophobic regions which are likely to act as points of attachment to the surface. In terms of native structure, they are fairly unique among proteins, and it is evident that their structures, when adsorbed are not typical of all proteins. Because casein (particularly β -casein) can spread rapidly it has high emulsifying power, so that relatively small amounts of casein can form stable emulsions. Other proteins are less flexible in their ability to rapidly cover an interface, and so casein is able to make emulsions with smaller droplet sizes than other proteins. Nisin is flexible and amphiphilic like casein, but lacks the aqueous solubility of casein.

CONCLUSIONS

Nisin is a cationic polypeptide that shows antibacterial activity against wide range of Gram-positive bacteria. It found wide application as preservative in food products, such as milk and canned food. It is also used as preservative in cosmetic ointments. Due to its amphiphilic properties, surface activity, flexibility, intermediate size between proteins that are used in food emulsions and small emulsifiers used in pharmaceutical emulsions, stability to heat and pressure extremes, very low toxicity and antibacterial activity, nisin might be a good alternative to emulsifiers currently used by the industry.

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CHAPTER 6

EFFICACY OF THE ANTIMICROBIAL PEPTIDE NISIN IN EMULSIFYING OIL IN WATER

ABSTRACT

Nisin is a small (3510 Da) cationic amphiphilic peptide that kills susceptible bacteria by insertion into membranes resulting in pore formation, and has found application as a “food grade” preservative in the food and beverage industries. The oil-in-water emulsifying activity of nisin is characterized here in using emulsion conductivity measurements. Evaluation of nisin as an emulsifier was accomplished by measuring its ability to stabilize dispersed oil droplets in water. Emulsions were formed after homogenization of a volume of nisin solution and corn oil; the electrical conductivity was measured continuously. Qualitatively, the higher the emulsifying activity of a given emulsifier, the lower the expected conductivity during homogenization. After homogenization, emulsion conductivity generally increases, reflecting that the dispersed oil droplets rise and coalesce to form a floating layer, and the rate of this increase provided a measure of emulsion stability. Nisin solutions at concentrations of 0.1, 0.5, 1, 5, and 10 mg/ml were used to prepare emulsions. Nisin showed significant emulsifying activity in comparison to Tween[®] 80 and β -casein. Nisin’s emulsifying activity was also found to be highly concentration and pH dependent.

INTRODUCTION

Emulsions are one of the major types of food structures, ranging from relatively simple liquids, such as milk and milk products to the structures found in comminuted meat products or ice creams (1). Many drugs are administered as emulsions in order to increase chemical stability, improve bioavailability over solid dosage forms, allow a liquid dosage form for poorly soluble drugs, or achieve drug targeting (2, 3). Proteins used effectively in food emulsions include the milk proteins (caseins, α -lactalbumin and β -lactoglobulin), egg proteins (from both egg white and yolk) and animal proteins, such as gelatin (1). Small emulsifiers used in pharmaceutical emulsions, such as Tween[®] 80, can cause systemic toxicity (4). For example, lipid microemulsions, used to improve dissolution and oral bioavailability of hydrophobic drugs, are thermodynamically stable, which requires the addition of a high percentage of emulsifiers. Consequently, they can have serious adverse effects, such as irritation of the intestinal mucosa and gastrointestinal bleeding upon oral administration, and hemolysis of red blood cells upon intravenous administration (4).

Nisin is a highly surface active molecule whose adsorptive properties were recognized as early as 1949 when it was noted that nisin in culture remained with *Lactococcus lactis* cells rather than in the culture broth at pH 6 (5). In 1951, Friedman and Epstein (6) recognized that nisin could adsorb onto glass test tubes, and that 2 U/ml could be released when sterile, fresh broth was added to the empty tubes. They also found that boiling in the presence of detergents and oxidizing agents was not sufficient to remove all the adsorbed nisin. Nisin adsorption at silanized silica and the retention of its antibacterial activity after adsorption was recently studied (7-9). It was observed that

nisin can adsorb to solid surfaces in a manner stable to rinsing, maintain activity and kill bacterial cells that have subsequently adhered. Nisin has an effect similar to cationic detergent effect on the bacterial cell membrane as leakage of UV absorbing material from treated bacterial cells was observed (11). Nisin can withstand activity loss during thermal processing and exposure to acidic environments and pressure extremes (12). These characteristics and others, such as non-toxicity, and antimicrobial activity (13) make it an attractive candidate for use as an emulsifier in food and pharmaceutical emulsions.

The main objective of this work was to determine if there is any oil-in-water emulsifying activity and emulsion stabilizing capacity of nisin, and compare the results with those of Tween[®] 80 (a commonly used nonionic emulsifier in food and pharmaceutical emulsions) and β -casein (an efficient food emulsion stabilizer).

MATERIALS

Pure nisin (about 5.0×10^7 IU/g) was obtained from Aplin and Barrett Ltd. (Dorset, U.K.). Tween[®] 80 (Lot No. 44H01211) and β -casein (Lot No. 25H9550) were purchased from Sigma Chemical Co. (St Louis, MO). Monobasic sodium phosphate monohydrate (Lot No. 77892KLJP), dibasic sodium phosphate heptahydrate (Lot No. 7914KJKA) and citric acid monohydrate (Lot No. 062777KMBX) were obtained from Mallinckrodt Specialty Chemical Co. (Paris, Kentucky). Sodium citrate (Lot No. 402346) was from J.T.T. Baker (Philipsburg, NJ). Corn oil (100% pure) was purchased locally (Hunt-Wesson, Inc. (Fullerton, CA)).

METHODS

Solution preparation

Nisin was dissolved in 0.01 M sodium phosphate monobasic (pH 4.5), to assure complete solubilization. Sodium phosphate dibasic (0.01 M, pH 9.1) was then added to the nisin solution to bring the pH to 7.4. Nisin solutions of pH 5.2 and 3.0 were prepared using citrate buffer (0.01 M). The same buffers were used to prepare β -casein and Tween[®] 80 solutions.

Emulsion conductivity measurement

An apparatus for measuring emulsion conductivity was used, consisting of a glass column connected to a conductivity cell (Cat. No. 018014, Orion Research Inc., Boston, MA). A schematic of the apparatus is shown in Figure 6.1. The design was adapted from Kato et al. (14) and described by Suttiprasit et al. (6). A small lab homogenizer (Biospec Products, Bartlesville, OK) was used to homogenize 6.7 ml of corn oil and 20 ml of emulsifier solution; the duration of homogenization was 1.5 min. All experiments were performed at room temperature (23-25°C). The electrical conductivity was recorded during homogenization and for 3.5 minutes after homogenization. The conductivity curves generally consisted of three regions (Figure 6.2): region a-b which is an immediate consequence of the homogenization and emulsification process; region b-c during which the conductivity is maintained low and quite constant as a result of formation of a stable oil-in-water emulsion; and an ascending linear region (c-d) due to emulsion instability.

Figure 6.1. Apparatus for measurement of emulsion conductivity

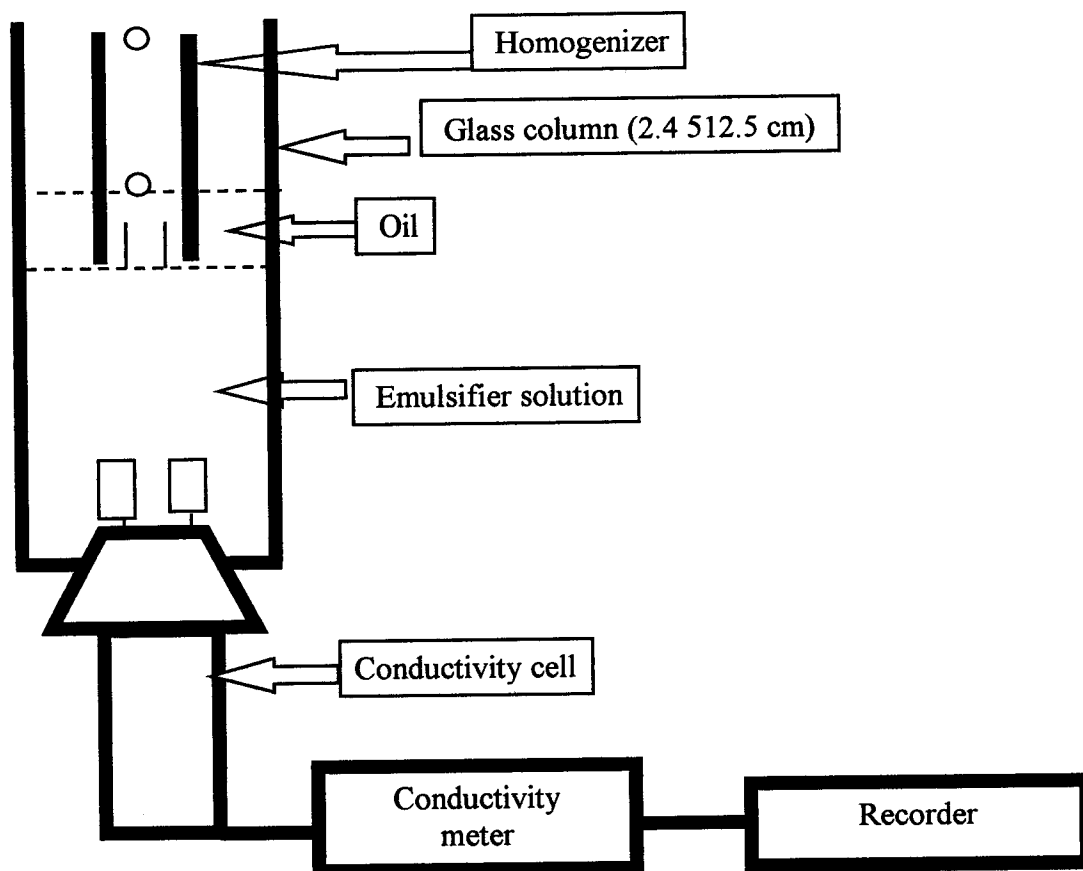
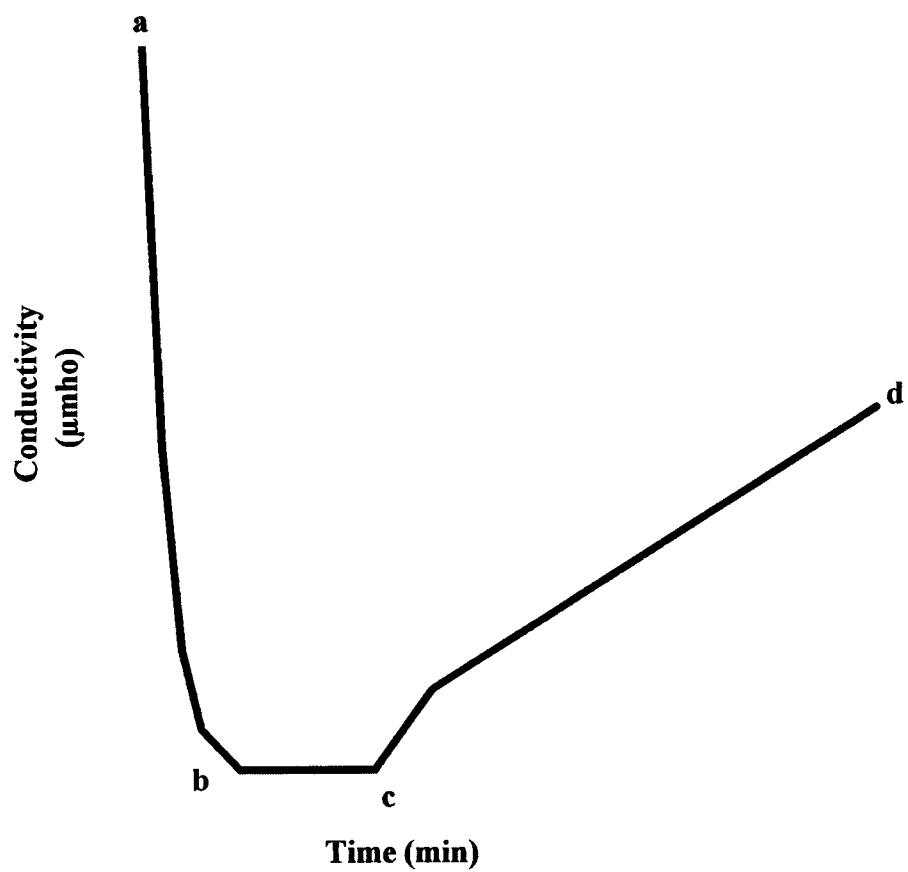


Figure 6.2. Emulsion conductivity curve divided into three distinct regions.



Determination of emulsion activity and emulsion stability

Both emulsion activity and emulsion stability were determined according to Kato et al. (14). The sharp initial drop in conductivity after homogenization is a result of mixing the nonconducting oil into the aqueous phase. This decrease depends on both the emulsion activity of a surfactant and its concentration: as a higher volume of oil is being dispersed, a lower conductivity is expected during homogenization. Accordingly, emulsion activity (EA) can be defined as the difference between the conductivity in the aqueous phase immediately before homogenization (C_p) and the lowest conductivity obtained during homogenization (C_0) (14):

$$EA = C_p - C_0 \quad [1]$$

Since the conductivity after homogenization would increase in proportion to the instability of the emulsion, the emulsion stability (ES) can be calculated using the linear portion of the conductivity curve after emulsification (14), or

$$ES = (C_p - C_0) \times \Delta t / \Delta C \quad [2]$$

where $\Delta t / \Delta C$ is the reciprocal of the slope of the linear portion of the conductivity curve after homogenization. Kato et al. (14) suggested that the nonlinear portion of the curve during the first 15 s after homogenization was due to the rise of foam formed during emulsification. Accordingly, this part was not included in calculating emulsion stability.

Image and particle size analysis

All samples for image and particle size analysis were taken from the bottom of the glass column at about 3.5 minutes after homogenization. Samples for analyzing the shape of flocculated and coalesced particles were drawn from the upper, separated layer of the emulsion. An incident light microscopy image analysis system (IAS) was used to record images of suspended oil droplets. The IAS is composed of a 486 IBM compatible computer interfaced to a Sony monitor and a Reichert Epistar incident light microscope to which a video camera (Cohu Inc., San Diego, CA) is attached. Image-Pro Version 2.0 (Media Cybernetics®, Silver Spring, MD) was used for digitizing images, and Visionplus-AT (Imaging Technology Inc., Bedford, MA) was used for image processing. Images were recorded under 40X objective. The average diameter of oil droplets in 10 images was taken as a measurement of particle size.

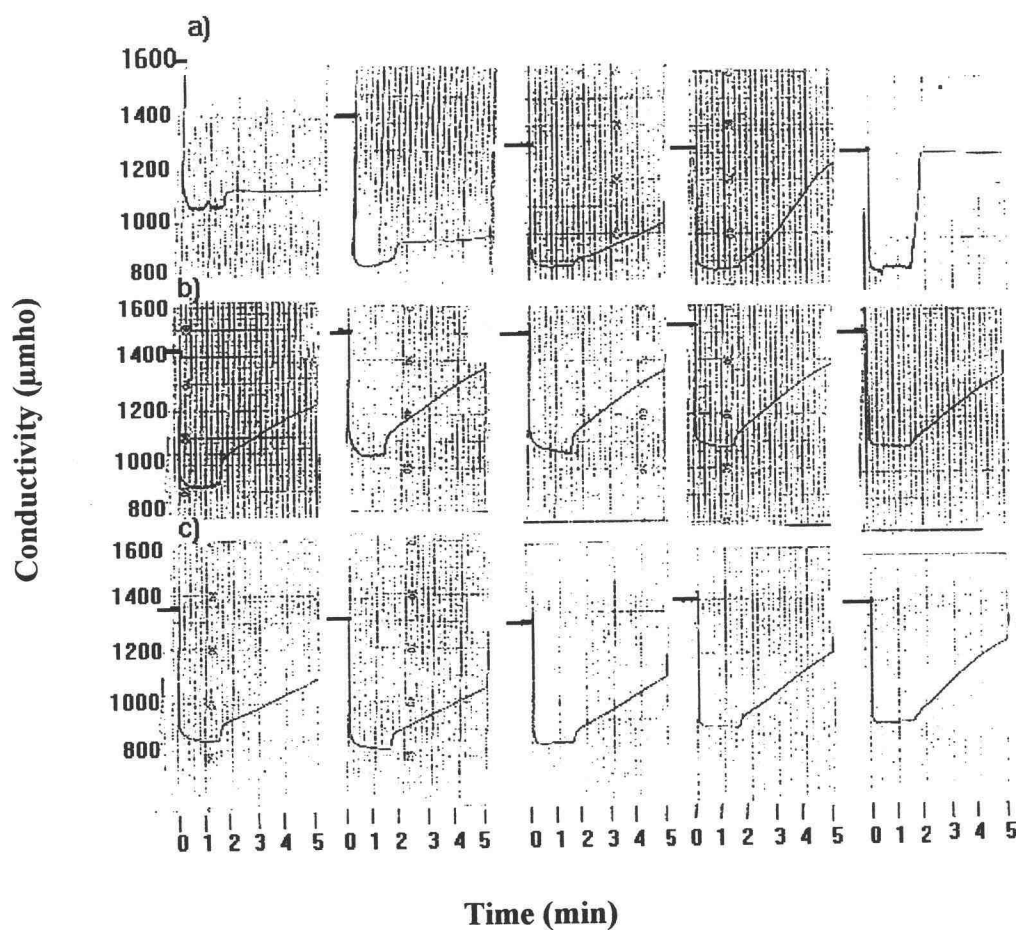
RESULTS AND DISCUSSION

Emulsion activity

Figure 6.3 shows that conductivity becomes time independent at about one minute after the onset of homogenization. The decrease in conductivity depends on the volume/area ratio of the dispersed phase.

At constant homogenization energy input, the amount of dispersed oil droplets and their particle size depend on emulsifier interfacial activity.

Figure 6.3. Emulsion conductivity curves for a) nisin b) β -casein and c) Tween[®] 80. Concentrations used from left for each emulsifier, 10.0, 5.0, 1.0, 0.5, and 0.1 mg/ml. The conductivity of each solution immediately before homogenization is indicated with an arrow on each curve.



The higher volume/area ratio of the dispersed phase during emulsification, the more emulsifier molecules required to be adsorbed at the newly created surfaces. Thus, the emulsion activity will depend on the emulsifier concentration in the bulk (15).

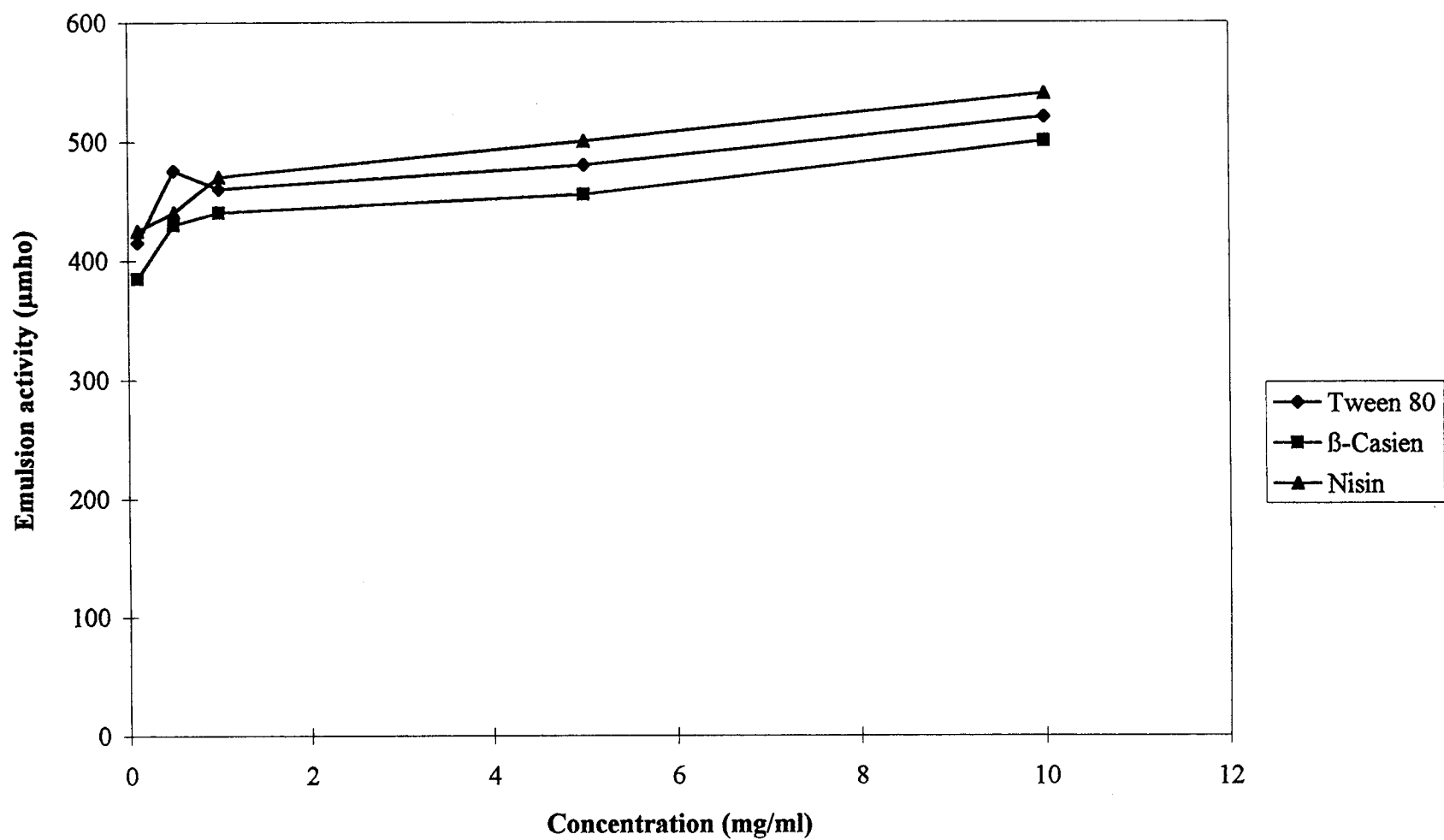
Figure 6.4 illustrates that the emulsion activity of each emulsifier increases with the increase in concentration; however, the differences in emulsion activity among the three emulsifiers are slight.

Emulsion stability

Emulsions are thermodynamically unstable systems, so after homogenization dispersed globules tend to flocculate or coalesce into aggregates or larger droplets. The increase in particle size facilitates the upward movement of the dispersed oil and the conductivity increases with time. The capacity of an emulsifier to stabilize an emulsion depends on both its concentration as well as its mechanism of stabilization. Adsorption of protein at the oil-water interface does not always lead to a stable emulsion; the protein must form a rigid interfacial layer. A thick layer of protein around the droplet will inhibit the thinning required to allow coalescence. Thick layers are likely to be mechanically strong, and they are also likely to prevent the close approach of particles through steric stabilization. Intuitively, a protein which makes strong contacts with its adsorbed neighbors will form a stronger layer than one which does not (1).

The slope of the linear portion of the conductivity curve after homogenization was observed to decrease with an increase in concentration (Figure 6.3). The effect of concentration on the rate of change in conductivity after homogenization is much more dramatic in the case of nisin than for Tween[®] 80 and β -casein. At a nisin concentration of

Figure 6.4. Effect of concentration on emulsion activity at neutral pH.



0.1 mg/ml, the conductivity was about equal to that before emulsification in less than 30 s, consistent with a highly unstable emulsion. But, emulsions stabilized by nisin showed only slight increase in conductivity after homogenization at 5 mg/ml relative to Tween[®] 80 and β -casein, and almost no increase in conductivity after homogenization at 10 mg/ml. The plots of emulsion stability vs. concentration (Figures 6.5a and 6.5b) are consistent these qualitative conclusions. It is not surprising that low concentrations of nisin do not form as stable an emulsion as low concentrations of the known emulsifiers (casein and Tween 80[®]). It is surprising the high concentration of nisin form more stable emulsions than equal concentrations of casein or Tween 80[®].

A schematic of the nisin molecule is given in Figure 5.1. Nuclear magnetic resonance (NMR) has been used to show that nisin adopts a kinked but rod-like conformation in DMSO (16). Modeled as a cylinder; the overall dimensions of the molecule are about 5 nm \times 2 nm (8). Van de Ven et al. (17) reported that nisin consists of two domains: the first is comprised of residues 3-19 and includes the first three lanthionine rings, and the second consists of the coupled ring system formed by residues 23 to 28. The two domains are connected by a flexible “hinge” region around methionine 21. Nisin has an amphiphilic character, with a cluster of bulky hydrophobic residues at the N-terminus and hydrophilic residues at the C-terminal end (17). The structural features of nisin now suggest that upon emulsion formation, its hydrophobic part can penetrate the oil, while its hydrophilic part would extend away from the interface into the bulk.

Rigid film formation at the oil-water interface, with steric and electrical stabilization of the dispersed droplets may explain the high emulsion stabilizing capacity

Figure 6.5a. Effect of concentration on emulsion stability at neutral pH.

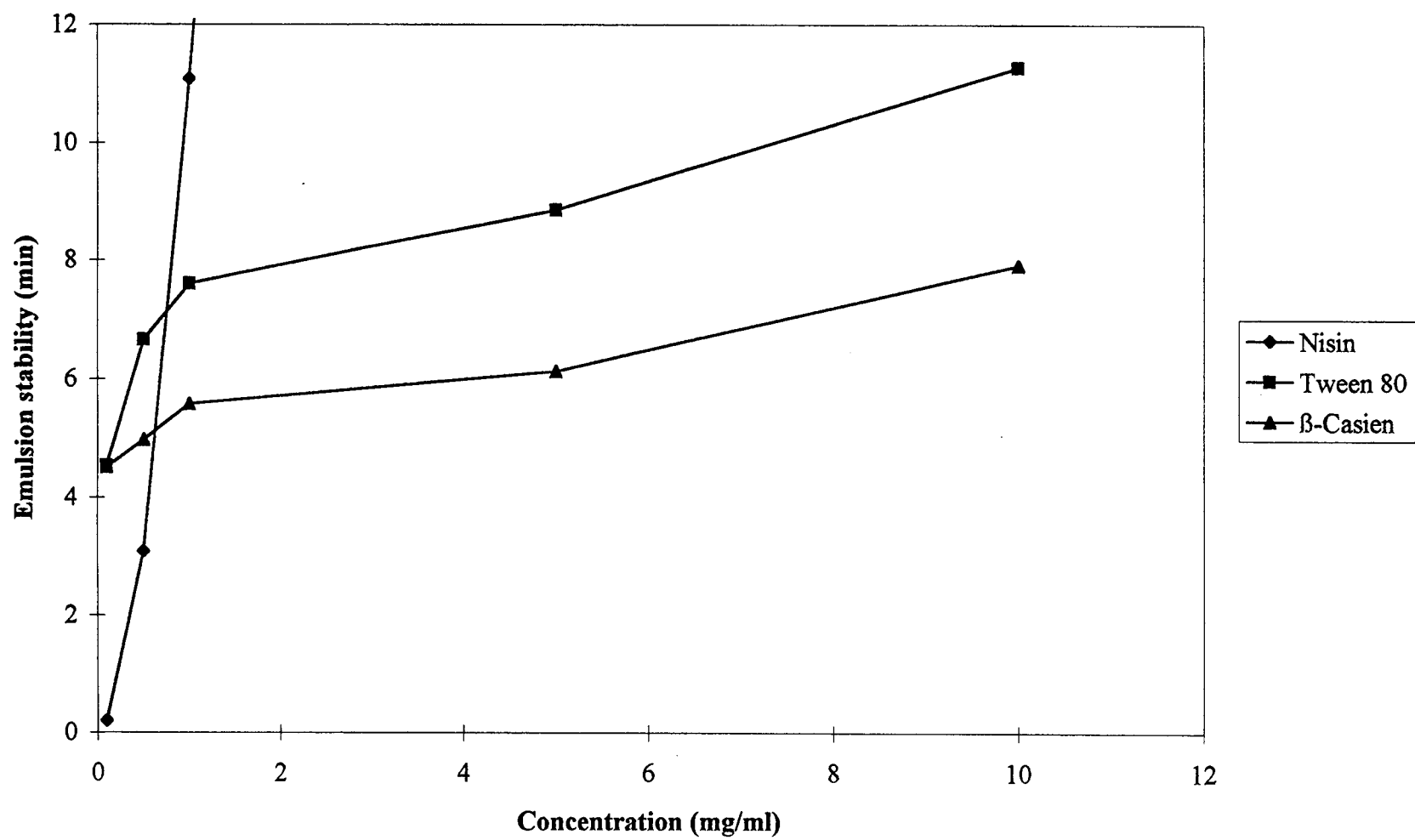
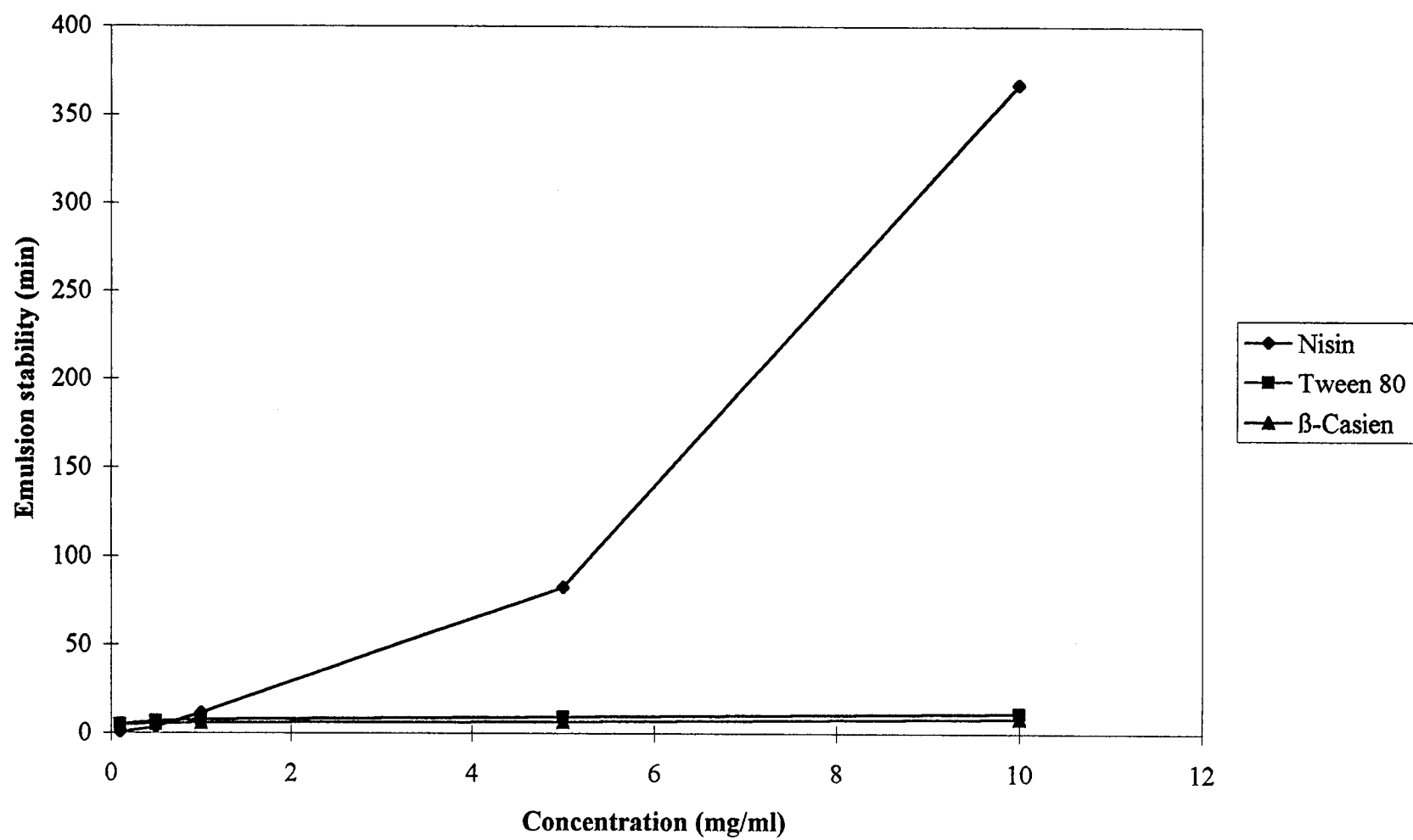


Figure 6.5b. Replot of Figure 6.5a with an expanded scale on the ordinate.



of nisin at high concentration. In contrast, the inability of nisin to stabilize emulsions at low concentration may possibly be due to incomplete coverage of the oil droplets.

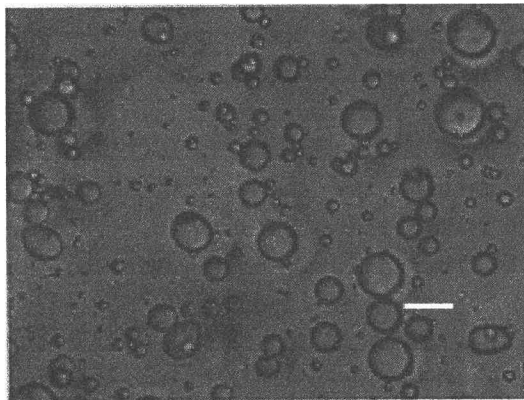
Image analysis

Dispersed oil droplets stabilized by Tween[®] 80 and β -casein were spherical under the microscope (Figures 6.6b and 6.6c). In the case of nisin at neutral pH, deformed oil droplets were observed in addition to the spherical ones. Under acidic conditions, nisin produced mostly spherical oil particles. The size of the deformed droplets ranged from about 2-4 μm (Figure 6.7a) to less than 0.5 μm (Figure 6.7b).

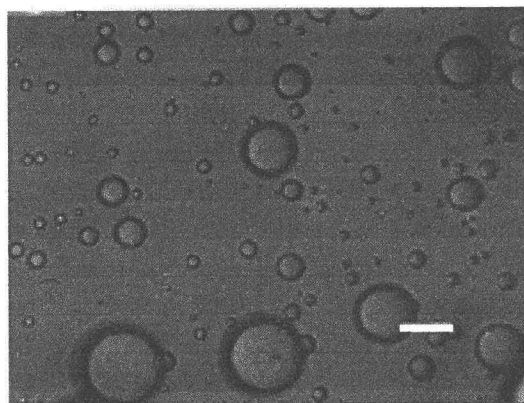
Magdassi and Vinetsky (18) studied the interaction of SDS and gelatin, and suggested the formation of an insoluble complex between these two components played the major role in microencapsulating oil-in-water emulsions. They stated that irregularity in droplet shape is a result of formation of a semisolid phase around the droplets. They found that the semisolid phase formed at specific SDS:gelatin concentration ratios and pH ranges. Accordingly, deformation of the oil droplets by nisin might be consistent with nisin associating with the oil and aqueous phases in a way to form a semisolid or gel-like structure. The percentage of nisin-stabilized spherical droplets present in an emulsion (Figure 6.6a) increased with increasing nisin concentration, indicating that any semisolid phase would be less likely to form at high nisin concentration. Samples from the oil-rich, separated, layer were examined under the microscope as well. At a nisin concentration of 0.1 mg/ml, a gel-like structure (Figure 6.8a) was observed. No gel-like structure was observed at higher concentrations, which is consistent with the thought that a semisolid phase is less likely to form at high interfacial concentrations of nisin.

Figure 6.6. Spherical oil droplets stabilized by: a) nisin; b) β -casein; and c) Tween[®] 80. Emulsifier concentration is 10 mg/ml. pH = 7.4. The bar shows 20 μ m.

a)



b)



c)

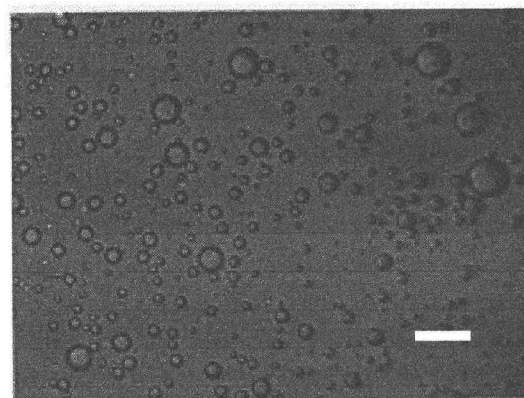
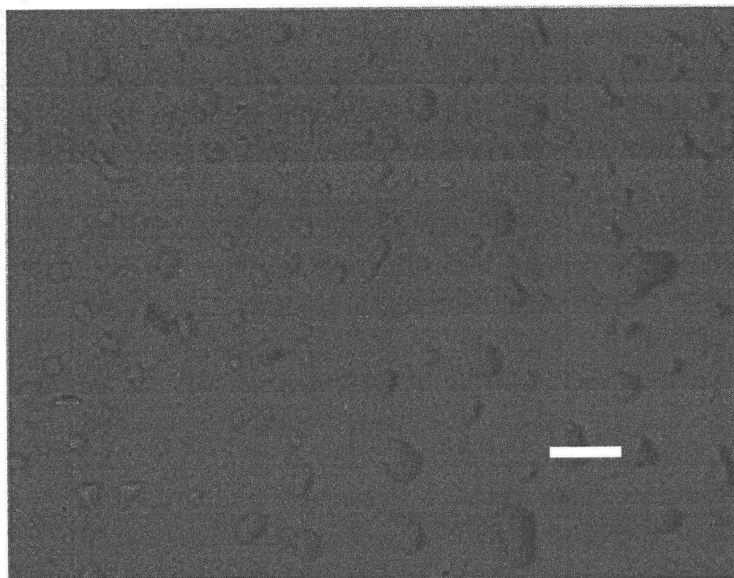


Figure 6.7. Deformed oil droplets stabilized by nisin at: a) 5 mg/ml; and b) 10 mg/ml. pH = 7.4. The bar shows 20 μm .

a)



b)

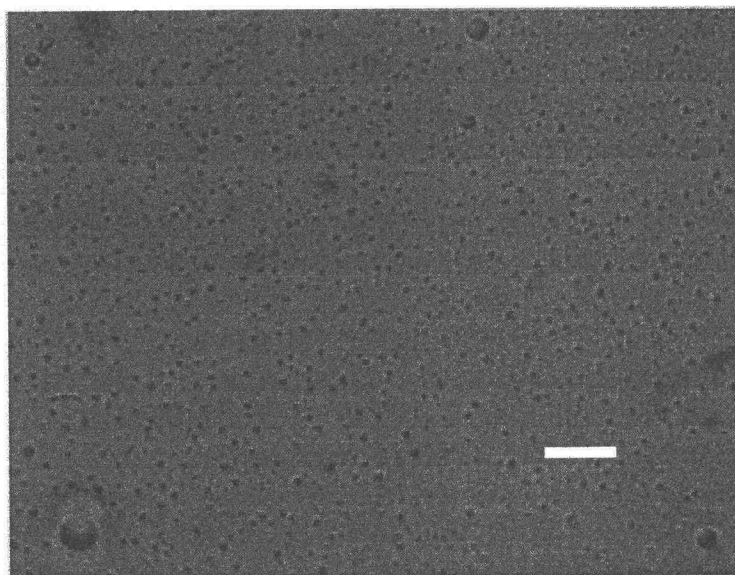
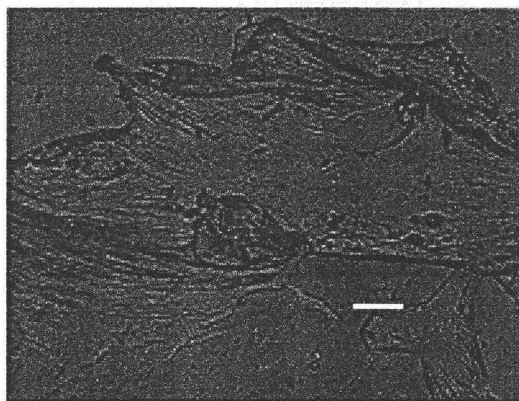
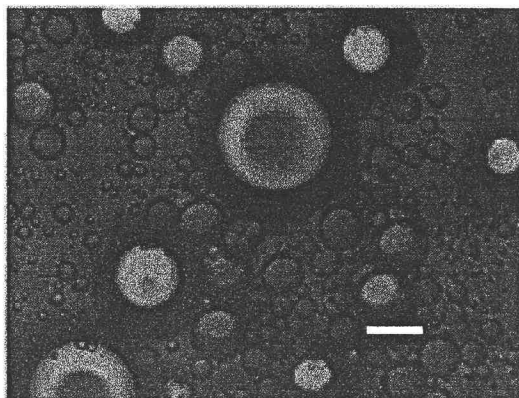


Figure 6.8. Aggregated oil droplets stabilized by: a) nisin; b) β -casein; c) Tween[®] 80. Emulsifier concentration is 0.1 mg/ml. pH = 7.4. The bar shows 20 μ m.

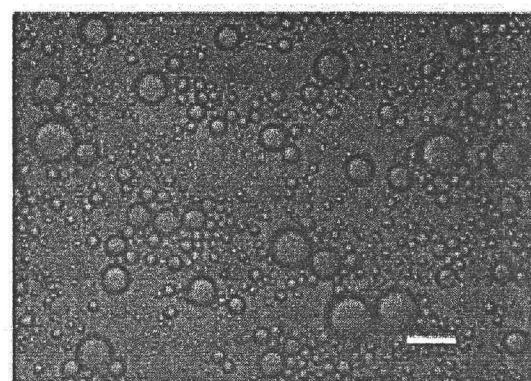
a)



b)



c)



Emulsions stabilized by nisin were examined for the presence of liquid crystals in polarized light; no optical pattern was seen. Samples from the oil-rich layer appeared as highly aggregated spherical particles when Tween[®] 80 or β -casein was used (Figures 8b and 8c).

Deformation is frequently encountered and leads to a reduced sediment or cream volume (19). The more rigid the interfacial film, the greater the resistance to deformation. On the other hand, the greater the size of the droplets and the density difference between the two liquid phases, the greater the tendency for deformation to occur (19). Oil droplets can distort into polyhedral "cells", resembling a foam in structure. This would result in a network of more-or-less planar thin films of one liquid separating cells of the other liquid (19). The stability of the system to coalescence would then depend on the stability to rupture of these films. At high nisin concentration, a continuous, rigid adsorbed film and small particle size may explain the increasing tendency of dispersed oil droplets to be spherical.

Particle size was not affected by Tween[®] 80 or β -casein concentration. The reason for this might be related to the relatively low stability of these emulsions. Even at the highest concentration tested, the shortest time required to prepare and examine a sample for particle size was greater than the magnitude of emulsion stability.

Effect of concentration on the emulsifying activity of nisin at acidic pH

Nisin stability, solubility and antibacterial activity are highly pH dependent; they decrease as the pH is increased (20). Therefore, the effect of pH on the emulsifying activity of nisin was studied. Figures 6.9a and 6.9b show that, contrary to what was found at neutral pH, the effect of nisin concentration on emulsion stability under acidic conditions is slight. They also show that with concentrations tested, nisin gives less stable emulsions than Tween[®] 80.

Because the net charge of a protein is least near its isoelectric point, it may be hypothesized that hydrophobic interactions between the protein and the oil surface would be less inhibited (1). Nisin has an isoelectric point in the alkaline range; thus, the net charge on its surface should be higher at acidic pH than at neutral pH. In addition, the decrease in nisin solubility with increasing pH indicates that hydrophobic associations are more favorable at neutral pH. Net charge may also affect the adsorbed mass by repulsion between adsorbed and adsorbing molecules at the interface. The adsorption of nisin to bacterial cells is pH-dependent (21), with a maximum in adsorption observed at pH 6.5, and less than half of that being adsorbed at pH 4.4 (22). The ability of nisin to form pore structures in the bacterial cell membrane depends on its ability to aggregate after adsorption. The higher antimicrobial activity of nisin at acidic pH than at neutral pH would suggest that self assembly is more favored at acidic pH. If we assume that the effect of pH on the aggregation of nisin molecules at the oil-water interface is similar to that at a bacterial cell membrane, fewer nisin molecules would be in monomeric form after adsorption at acidic than at neutral pH.

Figure 6.9a. Effect of concentration on emulsion stability at pH 5.2.

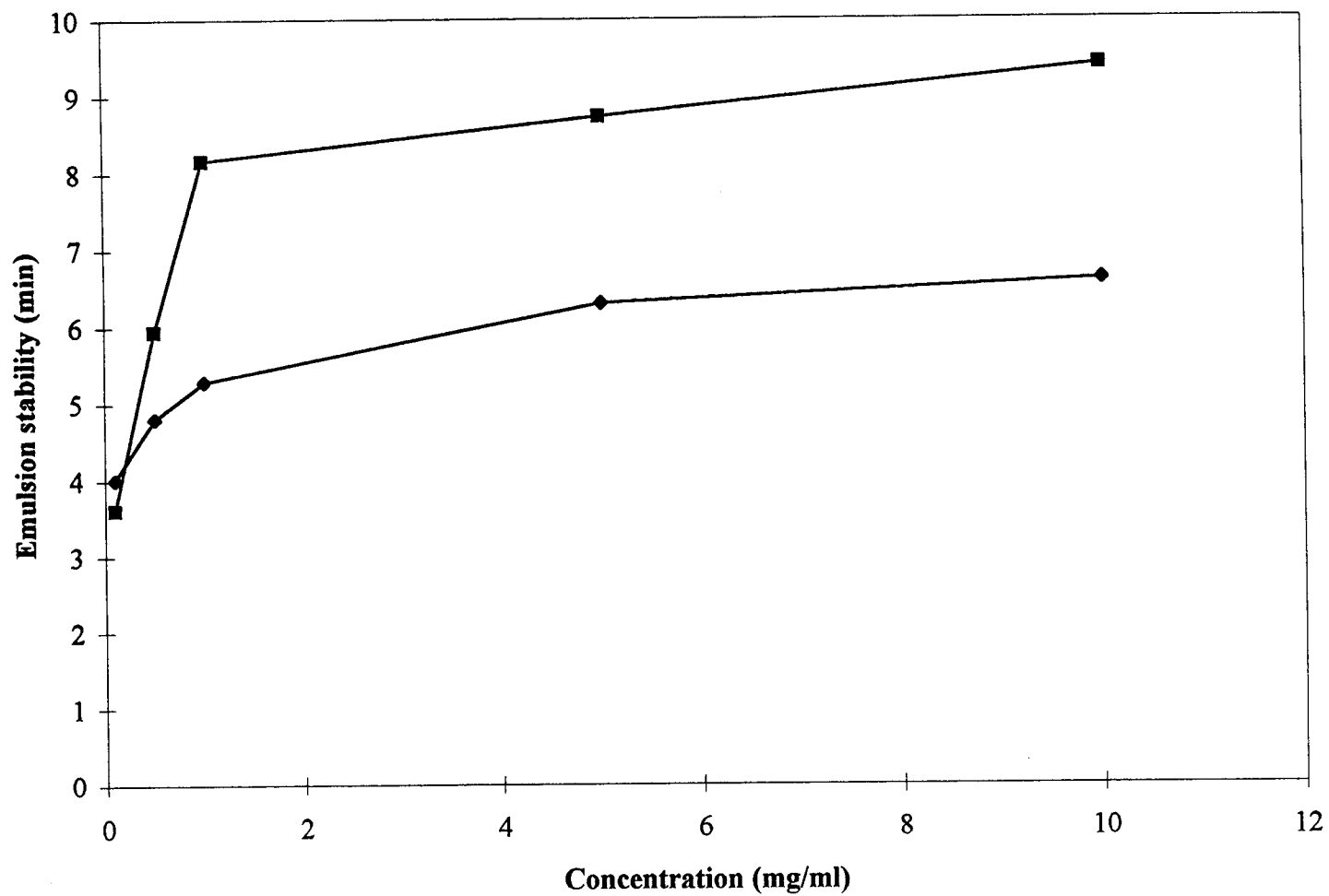
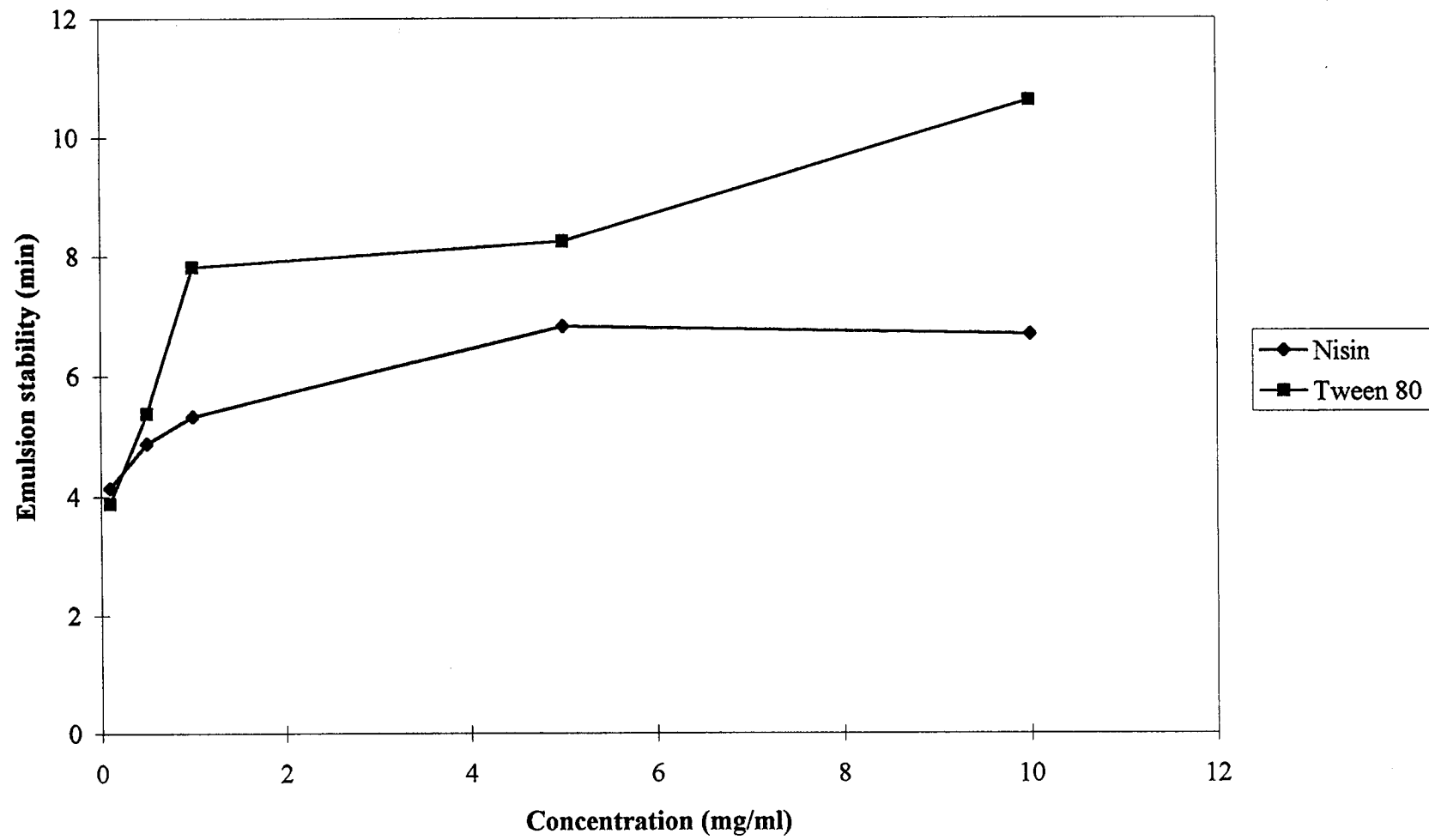


Figure 6.9b. Effect of concentration on emulsion stability at pH 3.0.



CONCLUSIONS

Emulsion conductivity measurements is a valuable tool to measure the emulsifying activity of surfactants. Nisin is an amphiphilic polypeptide with high surface activity. Nisin has high ability to stabilize oil in water emulsions in comparison to Tween 80 and β -casein. The emulsifying activity of nisin is highly concentration and pH dependent. It increases sharply with increasing concentration and pH. Adsorption of nisin into oil water interface can deform the structure of the dispersed oil droplets in oil in water emulsion, which might be due to gel formation.

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CHAPTER 7

INTERFACIAL TENSION KINETICS OF NISIN AND β -CASEIN AT AN OIL-WATER INTERFACE

ABSTRACT

The concentration- and time-dependence of interfacial pressure of nisin and β -casein at an n-hexadecane-water interface were evaluated using DuNoüy tensiometry. The two emulsifiers attained interfacial saturation at a bulk concentration of about 0.1 mg/ml, the reduction of the interfacial tension by nisin at that concentration being about equivalent to that of β -casein. The time dependence of interfacial tension recorded for each protein was described using two kinetic models. In the first, the reduction of interfacial tension with time was considered to be a result of molecular penetration into the interface followed by rearrangement. Nisin exhibited more rapid penetration and rearrangement at the interface than did β -casein. The second model allowed for the parallel, irreversible adsorption of protein into each of two states from solution, where state 2 molecules occupy a greater interfacial area and are more tightly bound than state 1 molecules. The extent of adsorption in state 1 and state 2 was determined to be highly concentration dependent for each protein; adsorption occurs mostly in state 1 at high concentration and mostly in state 2 at low concentrations. The effect of pH on interfacial activity of nisin was also studied and it was found that the interfacial activity of nisin decreases with decreasing pH.

INTRODUCTION

Proteins adsorb at air-water and oil-water interfaces, decreasing interfacial energy. The rate and extent of this decrease depend on many factors, including the size, charge, and flexibility of the adsorbing protein (1). Fluid interfaces differ from solid interfaces in allowing adsorbate molecules greater mobility at the interface and greater penetration into the nonaqueous phase (2). Thus, studying protein adsorption at gas-liquid and liquid-liquid interfaces is important for proper understanding of the ability of proteins to stabilize emulsions and foams in a variety of applications (2-7). Graham and Philips (1) suggested that, native molecules must first penetrate the air-water or oil-water interfaces, then unfold and rearrange for optimal packing. They also stated that adsorption is controlled by diffusion, a function of the size of the molecule. Thus, at low surface coverage, every protein molecule that arrives at the interface adsorbs spontaneously. Eventually, a steady state is achieved when the interface is saturated, and all of the molecules have rearranged to their preferred orientation (1,3).

The adsorption of proteins at air-water interface was described by a model that allows for tight adsorption of a first layer and loose packing of a second layer (4); in the first layer, protein adsorbs in different conformations with different occupied areas per adsorbed molecule, dependant upon surface concentration. Cho et al. (5) reported that the native and alkylated derivatives of bovine serum albumin occupy greater area at the air-water interface than that corresponding to molecular dimensions. They also found that the rate of surface pressure increase for these proteins was higher for higher bulk concentration at low times. In the classical 2-state theory of the globular protein unfolding transition (2), there are two protein structures: native and highly disordered.

However, it has now been established that an intermediate conformation can be present, termed the “molten globule”, defined as a protein with a native-like secondary structure but disordered (unfolded) tertiary structure (2). It was found that α -lactalbumin in the molten globule state (produced in the presence of EDTA) reduced the surface tension at the air-water and n-tetradecane-water interfaces more rapidly and to a lower level than the native protein (2). For globular proteins at the air-water interface, Farooq and Narsimhan (6) proposed that adsorbed segments are present in the form of “trains”. They concluded that the degree of unfolding of bovine serum albumin upon adsorption was greater than that of lysozyme, as they found that the number of segments per molecule increased linearly with the increase of surface concentration for bovine serum albumin, and was independent of surface concentration for lysozyme. Other studies suggested that protein molecules undergo conformational change during the adsorption process due to the interaction with the surface or during overcoming energy barrier to adsorption (8,9). In particular, free energy change of a protein molecule for conformational change to a denatured form, 5 to 14 kcal/mol, is comparable to the free energy for adsorption, 5 to 20 kcal/mol. Thus, the conformational change during adsorption is highly probable.

β -casein is a single chained, fibrous protein of molecular weight 24,000 that has no disulfide bonds (7). The N-terminal 21-amino acid sequence of β -casein contains one-third of the charged residues at pH 7, and this portion of the protein is highly solvated and flexible. The remainder of the molecule is nonpolar and very hydrophobic, making β -casein distinctly amphiphilic (7). Hunter et al. (7) modeled the adsorption of β -casein at the air-water interface using Langmuirian kinetics, defining an adsorption activation energy that depends on surface coverage. They found the isotherm exhibited two plateaus

in surface coverage. The isotherm was interpreted as indicating adsorption of a saturated layer of β -casein at low concentration followed by molecular reorientation and continued adsorption until the surface is once again saturated.

Nisin is a polypeptide (3510 Da) consisting of 34 amino acid residues (10). As far as the amino acid sequence is concerned, nisin possesses an amphiphilic character, with a cluster of bulky hydrophobic residues at the N-terminus and hydrophilic residues at the C-terminus (10). Nisin can withstand activity loss during thermal processing and exposure to acidic environments and pressure extremes (11). These characteristics and others, such as non-toxicity, high surface activity and antimicrobial activity (12) make it an attractive candidate for use as an emulsifier in food and pharmaceutical emulsions. The efficacy of nisin in emulsifying oil in water was evaluated (13) and found to be significant compared to β -casein and Tween[®] 80. The interfacial behavior of nisin and β -casein at hydrophilic and hydrophobic solid surfaces has been investigated using ellipsometry (14,15). Each of the proteins, when dissolved in single-protein solution, more favorably adsorbed at hydrophilic than at hydrophobic surfaces.

In this work, we evaluated the interfacial tension kinetics of nisin and β -casein at n-hexadecane-water interface using DuNoüy tensiometer. In order to account for the effect of conformational change during adsorption, the interfacial tension kinetic data of each protein were analyzed with reference to a two state mechanism that allows for protein to adsorb in structurally dissimilar forms.

MATERIALS AND METHODS

Materials

Pure nisin (about 5.0×10^7 IU/g) was obtained from Aplin and Barrett Ltd. (Dorset, U. K.). Tween[®] 80 (Lot No. 44H01211), n-hexadecane (Lot No. 105H3530) and β -casein (Lot No. 25H9550) were purchased from Sigma Chemical Co. (St Louis, MO). Monobasic sodium phosphate monohydrate (Lot No. 77892KLJP), dibasic sodium phosphate heptahydrate (Lot No. 7914KJKA) and citric acid monohydrate (Lot No. 062777KMBX) were obtained from Mallinckrodt Specialty Chemical Co. (Paris, Kentucky). Sodium citrate (Lot No. 402346) was from J.T. T. Baker (Philipsburg, NJ).

Solution preparation

Nisin was dissolved in 0.01 M sodium phosphate monobasic (pH 4.5), to assure complete solubilization. A suitable volume of sodium phosphate dibasic (pH 9.1, 0.01 M) was added to the solubilized nisin to bring the pH to 5.5, 6.5 or 7.4. Nisin solutions of pH 3.0 and 4.5 were prepared using citrate buffer (0.01 M). The same buffers were used to prepare β -casein and Tween[®] 80 solutions.

Interfacial tension measurement

A DuNoüy ring tensiometer (Model No. 70535, CSC Scientific Co., Inc., Fairfax, VA) was used to measure interfacial tension at the n-hexadecane-water interface. Immediately after gentle stirring for 45 s, 20 ml of emulsifier solution at a concentration

in the range of 1×10^{-6} to 1 mg/ml, was placed in a beaker (5 cm dia). The ring was immersed about 10 mm below the surface of the emulsifier solution, and this was followed by the addition of 20 ml n-hexadecane to the surface of the solution. The position (height) of the beaker was adjusted until the ring was in the interface and the apparent interfacial tension was measured (7). To account for the force needed to support the weight of the liquid clinging to the ring at the break point, the apparent interfacial tension was multiplied by a correction factor to get the true interfacial tension. All measurements were performed at room temperature (23-25°C). Before each measurement, the ring was cleaned by rinsing in benzene followed by rinsing in methylethylketone, and then heating in the oxidizing portion of the flame of an alcohol burner.

Interfacial pressure

Interfacial pressure, Π (mN/m), is the reduction of interfacial tension by a surfactant. The average of three interfacial tension readings between phosphate buffer (pH 7.4) and n-hexadecane was 52.4 mN/m. The interfacial pressure was obtained by subtracting the interfacial tension in the presence of a surfactant from this value.

RESULTS AND DISCUSSION

Effect of concentration on steady-state interfacial pressure

The concentration dependence of Π for nisin, β -casein and Tween[®] 80 is shown in Figure 7.1. Each emulsifier showed an increase in Π with increasing bulk concentration. The maximum reduction in interfacial tension was 42.2, 32.2 and 30.4 mN/m for Tween[®] 80, β -casein and nisin, respectively.

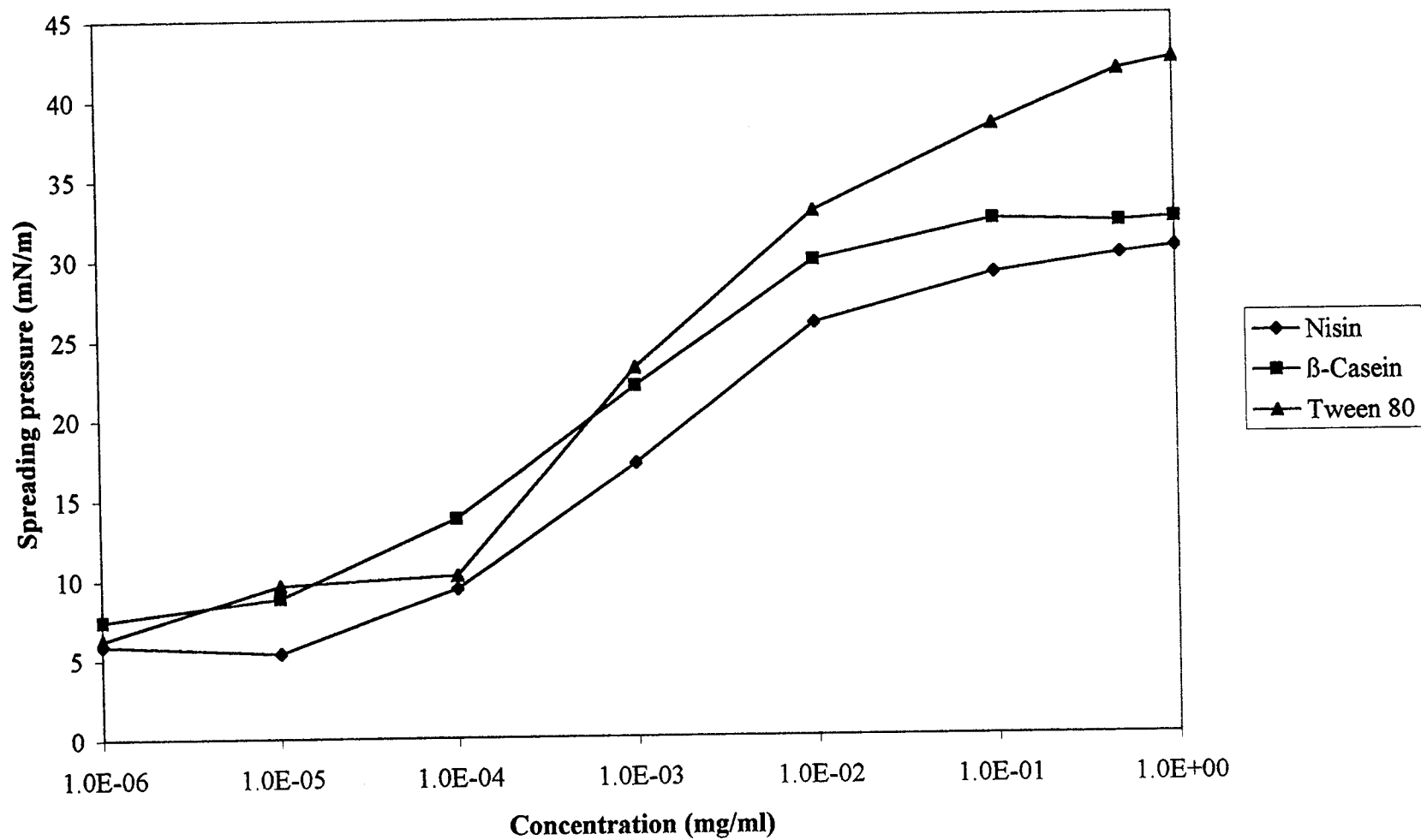
Gibbs adsorption equation can be used to calculate the surface excess concentration of an amphiphile. Applying Gibbs equation to protein adsorption is completely empirical, because molecules can do more than one point of attachment at an oil-water interface, and the area occupied by a single protein molecule can vary according to the interfacial concentration. It is of some qualitative interest, however, to apply the simple form of Gibbs adsorption equation to gain an expression for the surface excess concentration of a protein (Γ , mol/m²). That is, assuming adsorption equilibrium and that the activity of protein at the interface equals its concentration in the bulk, the equation can be written as (16)

$$\Gamma = (C_p/RT) \left(d\Pi/dC_p \right), \quad [1]$$

where R and T are the universal gas constant and absolute temperature, respectively. The change of spreading pressure with concentration can be fitted according to the following equation

$$\Pi = A_1 \ln (1 + A_2 C_p). \quad [2]$$

Figure 7.1. Effect of concentration on interfacial pressure.



Eq. [2] represents a model with two function parameters A_1 (mN/m) and A_2 (ml/mg). Suttiprasit et al (17) applied Eq. [2] to Π Vs concentration data of the proteins α -Lactalbumin, β -Lactoglobulin and Bovine Serum Albumin. The values of A_1 and A_2 estimated for nisin, β -casein and Tween[®] 80 along with the coefficient of determination (R^2) for each fit to Eq. [2] are listed in Table 7.1.

Table 7.1. Model parameter values estimated for Tween[®] 80, β -casein and nisin using Eq. [2].

Surfactant	A_1 (mN/m)	A_2 (ml/mg) $\times 10^{-6}$	R^2
Nisin	2.14	3.49	0.987
Tween [®] 80	3.34	0.64	0.995
β -Casein	2.39	1.28	0.996

Substitution of Eq. [1] into Eq. [2] yields

$$\Gamma = (A_1/RT)[(A_2C_p)/(1+A_2C_p)] \quad [3]$$

The adsorbed masses of the three emulsifiers were calculated according to Eq. [3] and listed in Table 7.2. The higher the value of A_1 and A_2 of a surfactant, the higher the saturation of the interface is attained with increasing bulk concentration. According to Table 7.2, nisin gives more saturation of the interface with increasing concentration than Tween[®] 80 or β -casein.

Table 7.2. Adsorbed mass (pmole/cm²) at oil-water interface calculated according to Eq. [3].

Concentration (mg/ml)	Adsorbed mass (pmole/cm ²)		
	Nisin	β -Casein	Tween [®] 80
10 ⁻⁶	67.30	54.26	52.71
10 ⁻⁵	84.15	89.58	116.57
10 ⁻⁴	86.31	95.81	132.64
10 ⁻³	86.53	96.49	134.49
10 ⁻²	86.56	96.55	134.68
0.1	86.56	96.56	134.70
0.5	86.56	96.56	134.70
1	86.56	96.56	134.70

Time dependence of surface pressure

Empirical analysis

Figures 7.2, 7.3 and 7.4 show time dependence of Π for Tween[®] 80, β -casein and nisin (solid lines for the higher concentration data of nisin and β -casein represent fits to a model that will be discussed in the next section). The three emulsifiers attained a steady state interfacial pressure, at all concentrations, after about 2 hours. For single protein solutions of β -casein, BSA and lysozyme, Graham and Philips (1) suggested that the rate

Figure 7.2. Time dependence of interfacial pressure for nisin as a function of concentration.

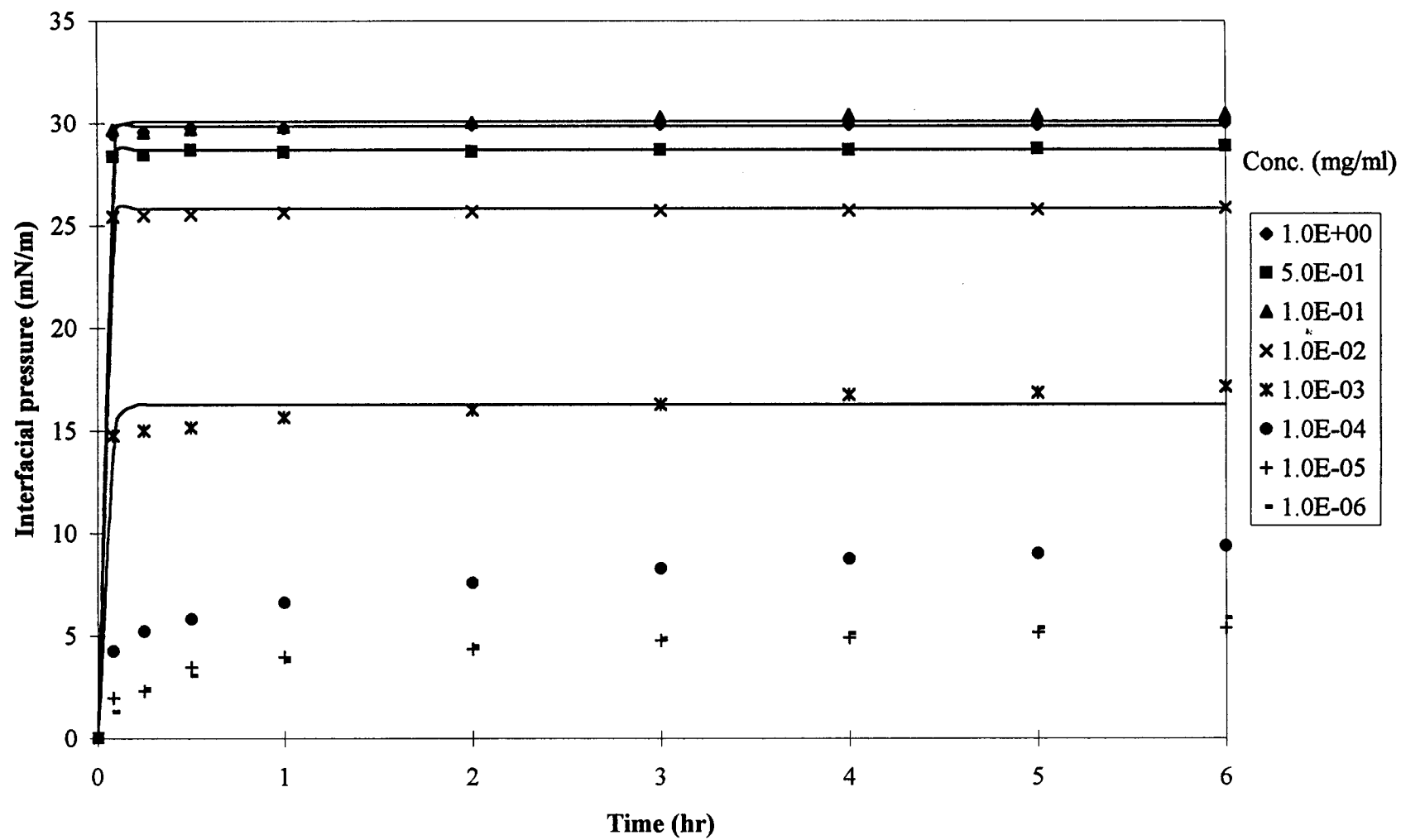


Figure 7.3. Time dependence of interfacial pressure for β -casein as a function of concentration.

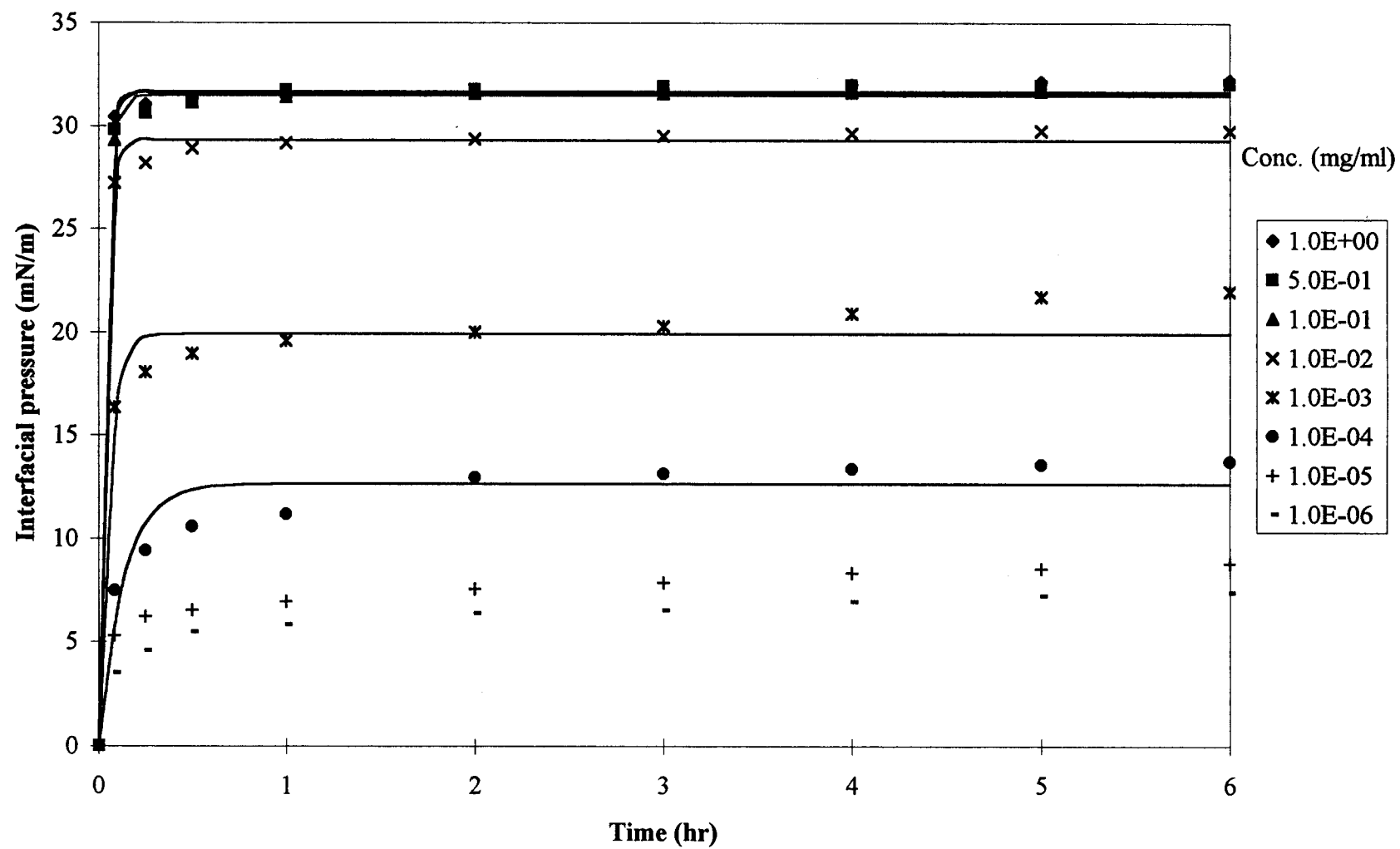
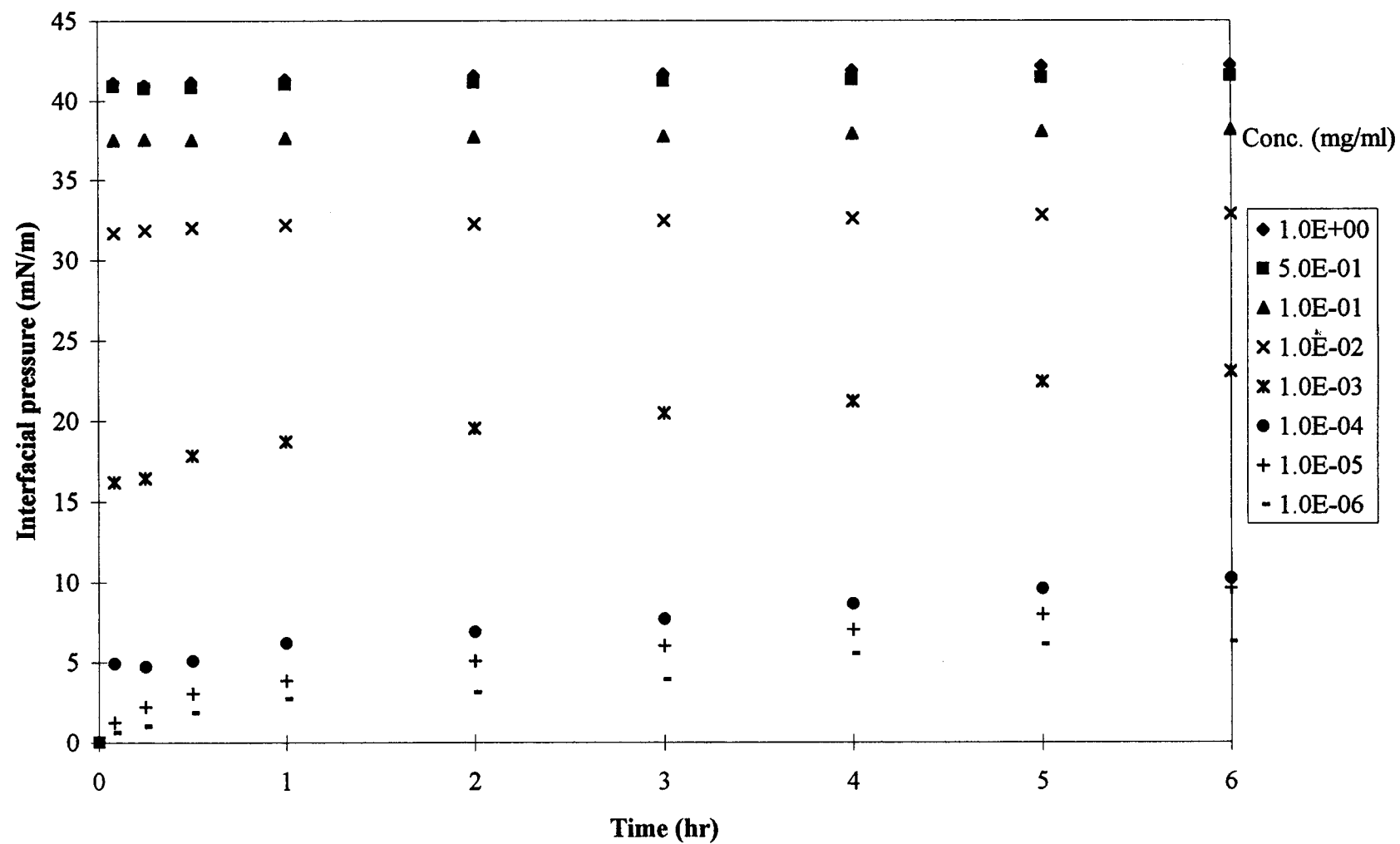


Figure 7.4. Time dependence of interfacial pressure for Tween 80 as a function of concentration.



of change of interfacial pressure can be defined with reference to two kinetic regions, each characterized by a first-order rate constant. The first region, the adsorption period, is one in which both adsorbed mass and interfacial pressure are observed to increase. The other region, the rearrangement period, is characterized by attainment of a plateau in adsorbed mass while the interfacial pressure continues to increase. Small emulsifiers, such as Tween[®] 80, can do further orientation at the interface after adsorption to optimize the hydrophilic and hydrophobic interactions, which results in a gradual reduction in interfacial tension with time (18). Caseins, particularly β -casein, possess relatively small amounts of regular structure, and are conformationally mobile, enabling them to readily optimize their configuration at the interface. In addition, they possess extensive hydrophobic regions (as can be estimated from their secondary structure), which are likely to act as the points of attachment to the interface (19). Globular proteins must denature in order to optimize their interaction (19). Also, adsorbed proteins can interact with each other through intermolecular bonds (H-bonds, salt bridges, and disulfides) which are not possible for small molecules (19). Rates of adsorption and conformational change can be represented by the first order equation:

$$\ln (\Pi_{\infty}-\Pi_t)/(\Pi_{\infty}-\Pi_0) = -kt, \quad [4]$$

where Π_{∞} , Π_t and Π_0 are the surface pressure values at steady state, at any time t , and at $t = 0$, respectively, and k is a first-order rate constant. In order to get an accurate estimate of the rate of penetration and conformational change. Eq. [4] should be applied in the absence of diffusion control. Graham and Philips (1) reported that at a bulk concentration

of 0.001 mg/ml, changes in Π for BSA were diffusion controlled for about 1 hr. After this period, the surface coverage was such that an energy barrier to adsorption made the rate of adsorption lower than that predicted by the rate of diffusion. When the rate of adsorption equals the rate of diffusion, the total surface concentration of molecules can be estimated by $2C_p(Dt/\Pi)^{1/2}$, where D is the diffusion coefficient. At 0.1 mg/ml, adsorption should be diffusion controlled for much less than 5 minutes, which was the time for the first interfacial tension measurement in this study. Fitting the interfacial kinetic data according to Eq. [4] usually yields more than one linear segment. The slope of the first linear segment is considered as an adsorption rate constant, while the slope of the second or third segment is interpreted as a rearrangement rate constant.

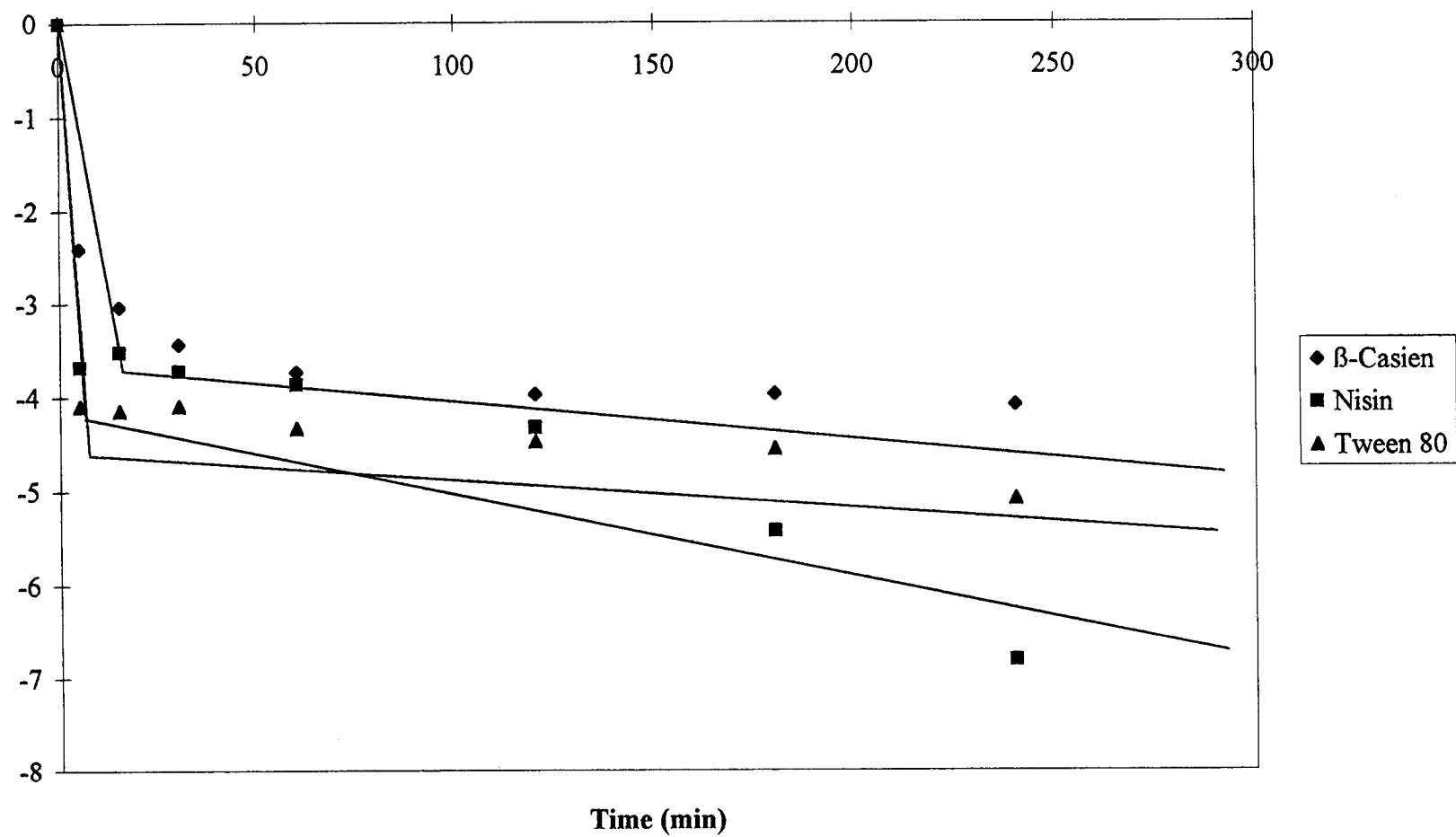
Figure 7.5 shows that each emulsifier has two distinct linear regions. The first-order rate constants estimated in each region, along with the coefficient of determination for each line, are shown in Table 7.3.

Table 7.3. First order constants associated with adsorption (k_a) and rearrangement or conformational change (k_r).

Emulsifier	$k_1 (10^{-2}\text{min}^{-1})$	$k_2 (10^{-2}\text{min}^{-1})$
Nisin	73.64 (1.00)	1.23 (0.93)
β -Casein	18.29 (0.754)	0.322 (0.749)
Tween [®] 80	82.59 (1.00)	0.538 (0.92)

Note. The coefficients of determination estimated for each line are shown in parenthesis.

Figure 7.5. Interfacial pressure data plotted according to Eq. (6).



The order of penetration rate constant of the three emulsifiers is Tween[®] 80 > nisin > β -casein. The rate of adsorption of a molecule from the bulk into interface is controlled by its size, hydrophobicity and concentration. The increase in concentration and hydrophobicity results in an increase in adsorption rate, while as the molecular size increases the adsorption becomes slower (1). Tween[®] is a small emulsifier with a molecular weight of 1,309.68, while β -casein is a giant protein with a molecular weight of 24,000 and is known to form aggregates in aqueous solution, particularly at high concentrations. Nisin is a polypeptide with molecular weight of 3,510, however, there is an evidence that dimers and tetramers occur having weights of 7,000 and 1,4000 (20,21).

Table 7.3 shows that nisin can achieve stable film at an oil-water interface much faster than β -casein and Tween[®] 80. A schematic of the nisin molecule is given in Figure 5.1. Van de Ven et al. (22) reported that nisin consists of two domains: the first is comprised of residues 3-19 and includes the first three lanthionine rings, and the second consists of the coupled ring system formed by the residues 23 to 28. The two domains are connected by a flexible “hinge” region around methionine 21. Nisin has an amphiphilic character, with cluster of bulky hydrophobic residues at the N-terminus and hydrophilic residues at the C-terminal end (22). The presence of a flexible “hinge” between hydrophilic and hydrophobic domains of nisin together might allow for rapid hydrophilic and hydrophobic associations at an oil-water interface.

Analysis with reference to a kinetic model

Wang and McGuire (23) applied a kinetic model to describe the spreading pressure of T4 lysozyme solutions, allowing protein to be adsorbed structurally and functionally dissimilar. State 2 molecules are unfolded to some extent and more tightly bound to the surface than those in state 1; also the area occupied by a state 2 molecule (A_2) is larger than that occupied by a state 1 molecule (A_1). The same adsorption mechanism can be applied to protein adsorption at an oil-water interface, and is illustrated in Figure 7.6. Rate constants k_1 and k_2 describe adsorption into state 1 and state 2 respectively. Neglecting the influence of diffusion, equations describing the time-dependent fractional surface coverage of protein in each of the two states (θ_1 and θ_2) shown in Figure 7.6 can be solved, such that

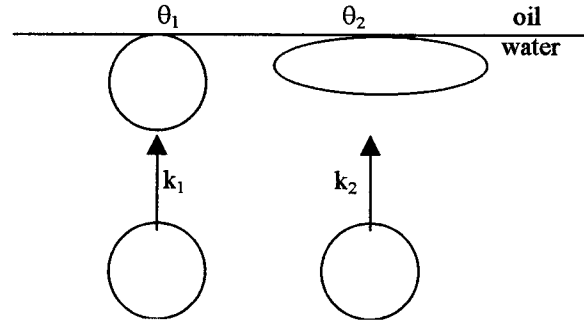
$$\theta_1 = 1/(1 + ak_2/k_1)[1 - \exp(-k_1C - ak_2C)t] \quad [5]$$

and

$$\theta_2 = [(k_2/k_1)/(1 + ak_2/k_1)][1 - \exp(-k_1C - ak_2C)t], \quad [6]$$

where a is A_2/A_1 , and C (mg/ml) is the bulk protein concentration.

Figure 7.6. A simple mechanism for protein adsorption at an oil-water interface.



Defining Γ_{\max} as the maximum adsorbed mass of molecules allowable in a monolayer, and θ_1 and θ_2 as mass of state 1 and state 2 molecules (mg/m^2) adsorbed at any time, respectively, divided by Γ_{\max} , the adsorbed mass of molecules allowable in a monolayer in at any time, Γ (mg/m^2), can be given by

$$\Gamma = \Gamma_{\max}(\theta_1 + \theta_2), \quad [7]$$

and when the surface is covered,

$$\theta_1 + a\theta_2 = 1. \quad [8]$$

The contribution of reduction in interfacial tension is different between state 1 and state 2; more reduction per interfacial area unit is attained with the adsorption in state 2. Π_1 and Π_2 are defined as the interfacial pressure when the interface is covered entirely by state 1 and state 2, respectively, and b is Π_1/Π_2 . The maximum interfacial pressure

measurable would correspond to a monolayer of state 2 molecules; i.e., $\Pi_{\max} = \Pi_2 > \Pi_1$.

The interfacial pressure at any time is therefore given by

$$\Pi = \Pi_{\max}(b\theta_1 + a\theta_2). \quad [9]$$

A model for the spreading pressure at any time can be obtained by substituting Eqs. [5] and [6] into Eq. [9]:

$$\Pi = \Pi_{\max}[(b + ak_2/k_1)/(1 + ak_2/k_1)] \times [1 - \exp(-k_1C - ak_2C)t]. \quad [10]$$

In order to solve for k_1C and k_2C in Eq. [10], the values of a , Π_{\max} , and b should be known or approximated. McGuire et al. (24) estimated the fraction of state 2 molecules present in a monolayer formed on hydrophobic silica (i.e., $\theta_2/(\theta_1 + \theta_2)$, evaluated at steady state) for wild type T4-lysozyme, and the mutants I3 and I3W. In a related study, Wang and McGuire (23) assumed air-water interface as an ideal hydrophobic surface and the fraction of state 2 molecules estimated for these proteins at hydrophobic silica to be similar to that at the air-water interface. They calculated Π_{\max} and b of these mutants using Eq. [10] applied at steady state. Because we did not have mutants for nisin or β -casein, b values of both proteins could not be calculated. If we assume as a first approximation that adsorption in states 1 or 2, while occupying different interfacial areas, would result in the same interfacial tension reduction, Eqs. [9] and [10] become

$$\Pi = \Pi_{\max}(\theta_1 + \theta_2), \quad [11]$$

and

$$\Pi = \Pi_{\max}[(1 + k_2/k_1)/(1 + ak_2/k_1)] \times [1 - \exp(-k_1C - ak_2C)t], \quad [12]$$

respectively.

Based on molecular dimensions, 20.5 × 20.5 × 50 Å for nisin (23) and 14.6 × 5 × 14.6 × 175 Å for β-casein (7), the parameters A_1 and A_2 can be estimated as the interfacial area occupied by adsorbed “end on” and “side on” molecules respectively, such that a is 2.50 for nisin and 11.99 for β-casein. The maximum measured reduction in interfacial tension (32.2 for β-casein and 30.4 for nisin) provide an estimate for the theoretical Π_{\max} .

The values of k_1C and k_2C for nisin and β-casein were estimated according to Eq. [12] and are listed in Table 7.4. In general, we can see that the rate of adsorption in state 1 decreases with decreasing concentration, while the rate of adsorption in state 2 increases with decreasing concentration. At high bulk concentration, the ratio of the interfacial area to the total number of molecules available for adsorption is very small, and saturation of the interface is attained in a very short period of time. This suggests that the sub-layer would be crowded, with only little uncovered interface being available as adsorption progresses. Alternatively, the possibility for a protein molecule to extend its conformation and be adsorbed in state 2 would be maximized in a dilute solution.

Table 7.4. Rate constants k_1C and k_2C , based on a calculated from molecular dimensions and fitting interfacial pressure kinetic data to Eq. [12].

Concentration (mg/ml)	Nisin		β -Casein	
	k_1C^a	k_2C^a	k_1C^a	k_2C^a
1	50.952	0.687	38.019	0.060
0.5	50.001	2.164	33.378	0.057
0.1	50.591	0.405	31.197	0.069
0.01	41.991	5.947	28.477	0.263
0.001	6.388	9.340	11.290	0.645
0.0001	-	1.972	2.518	0.413
0.00001	-	1.518	1.984	0.810
0.000001	-	1.011	0.755	0.410

^a ml/mg.min

- Negative value was obtained.

An experimentally determined “a” may allow for more accurate estimation of adsorption in states 1 and 2 using Eq. [12] than what was estimated using the molecular dimensions. The isotherms of the steady-state interfacial pressure versus the bulk concentration (Figure 7.1) appear to be sigmoidal with upper and lower critical concentration. The interfacial pressure does not change very much below the lower critical concentration, probably because adsorption occurs only in state 2. The lower critical concentrations were specified as $1.5 \cdot 10^{-4}$ mg/ml for nisin and $1.5 \cdot 10^{-5}$ mg/ml for β -casein. We assume that adsorption below these critical concentrations occurs in state 2 only, such that

$$\frac{d\theta_2}{dt} = k_2 C [1 - a\theta_2], \quad [13]$$

or

$$\Pi = \Pi_{\max} / a [1 - \exp(-k_2 / a) C t]. \quad [14]$$

a values obtained from Eq. [14] at the lower critical concentration (4.183 for β -casein and 3.718 for nisin) were used in estimating $k_1 C$ and $k_2 C$ for the two proteins according to Eq. [12] at concentrations higher than the lower critical concentration. The interfacial tension kinetic data, along with their fit to Eq. [12], are shown in Figures 7.1 and 7.2. The plots of $k_1 C$ and $k_2 C$ versus log concentration for both proteins (Figures 7.7 and 7.8) are consistent with the tendency for more adsorption in state 2 with the decrease in concentration. Figures 7.9 and 7.10 show that k_1 and k_2 decrease with increasing concentration. Guzman et al. (4) modeled the dependence on surface concentration for adsorption rate constants in terms of activation energies for adsorption and desorption. Accordingly, k_1 and k_2 might be best represented in the form $k_i = k_{i0} \exp(-E_{ai}/RT)$, where E_a , the activation energy for adsorption, is considered to be surface coverage dependent.

The results in our study suggest that the activation energy for protein adsorption increases with increasing surface concentration of nisin and β -casein; consequently, the ease with which a protein molecule adsorbs should decrease with increasing surface concentration. Hunter et al. (7) found that for β -casein, at low concentrations ($<10^{-3}$ mg/ml), adsorption at air-water interface was “cooperative” becoming easier as the

Figure 7.7. Adsorption rates k_1C and k_2C (ml/mg.min) for nisin as a function of concentration, based on fitting interfacial pressure kinetic data recorded to Eq. [12].

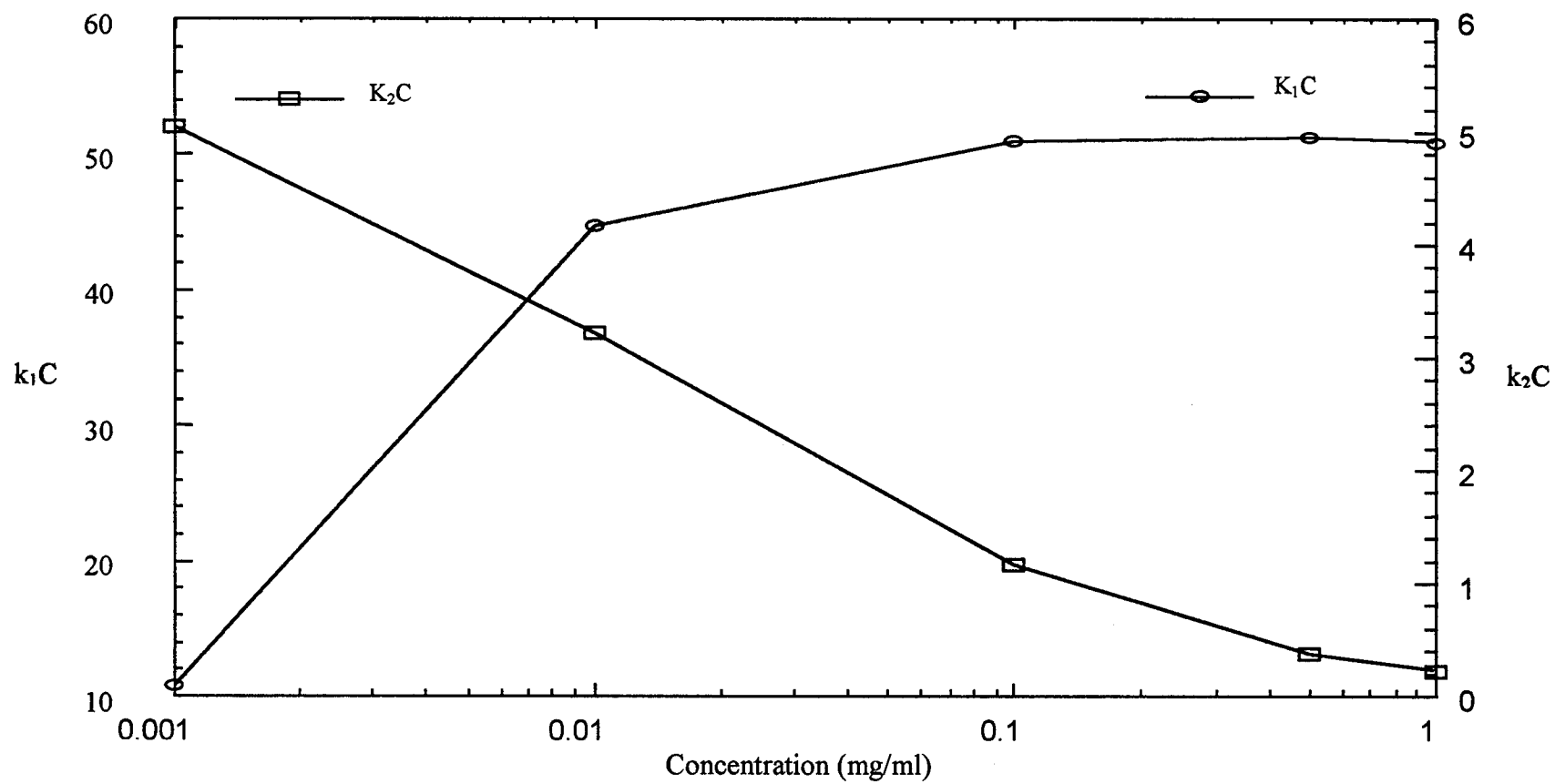


Figure 7.8. Adsorption rates k_1C and k_2C (ml/mg.min) for β -casein as a function of concentration, based on fitting interfacial pressure kinetic data recorded to Eq. [12].

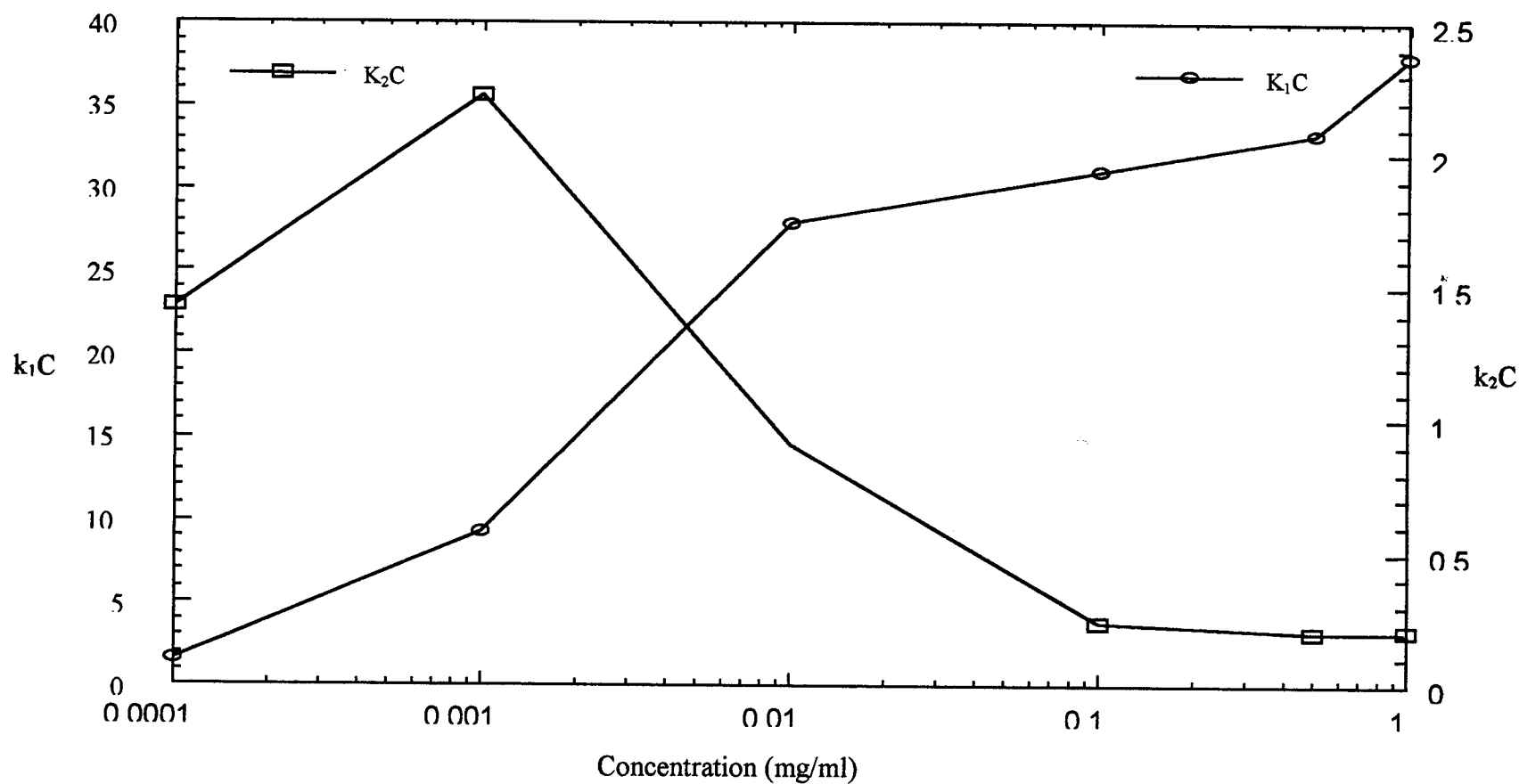


Figure 7.9. Adsorption rates k_1 and k_2 (ml/min) for nisin as a function of concentration, based on fitting interfacial pressure kinetic data recorded to Eq. [12].

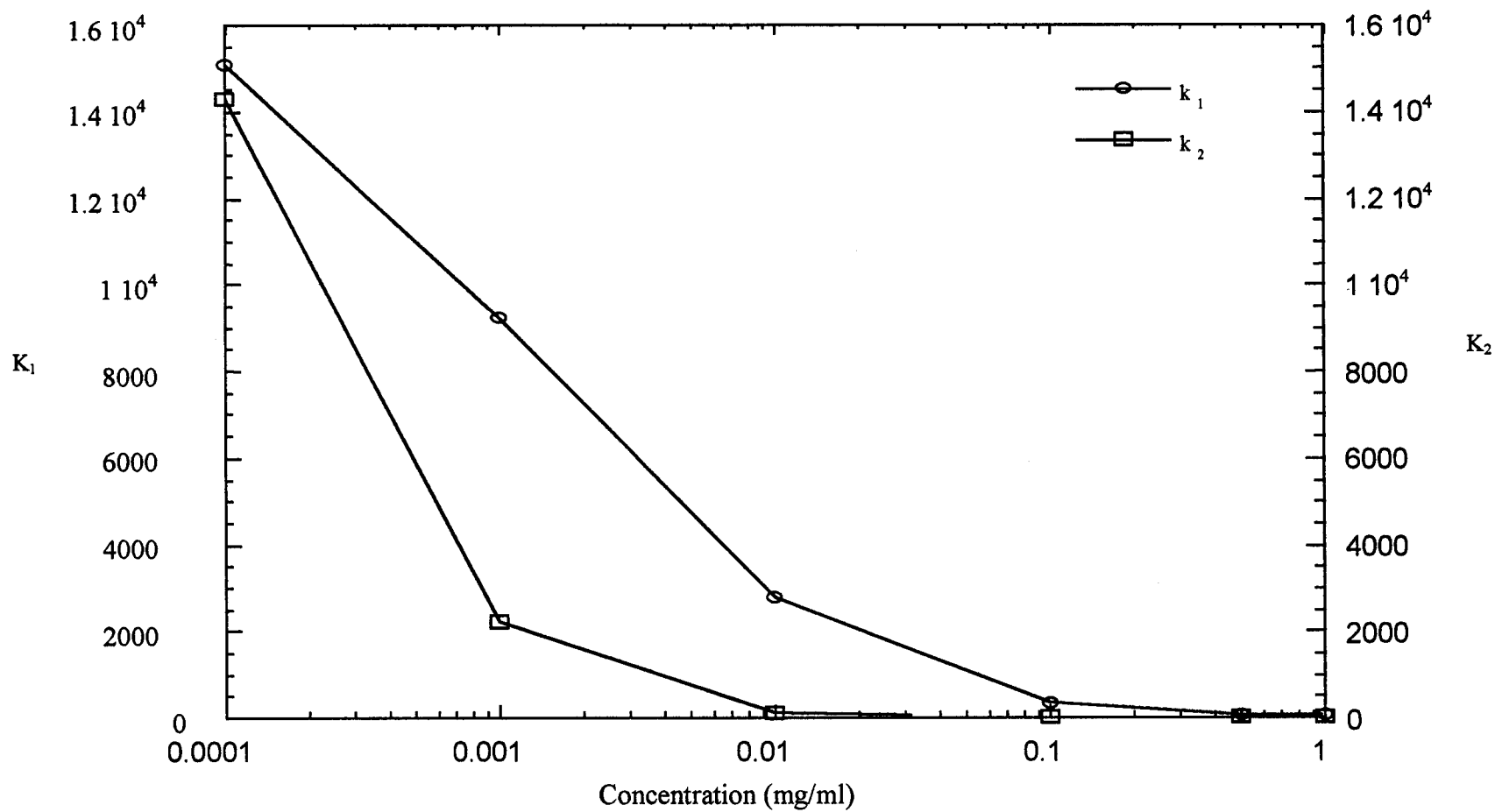
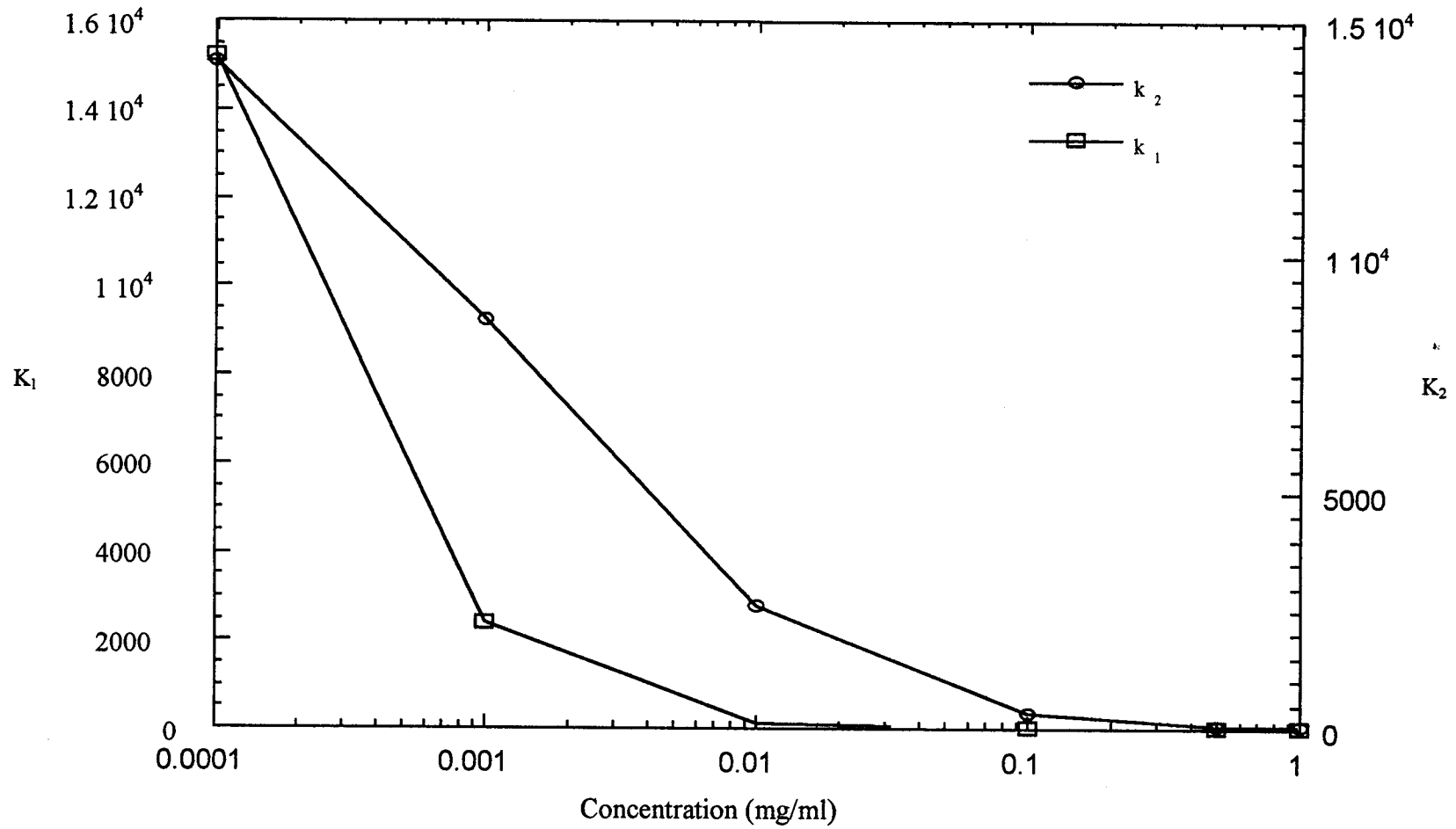


Figure 7.10. Adsorption rates k_1 and k_2 (ml/min) for β -casein as a function of concentration, based on fitting interfacial pressure kinetic data recorded to Eq. [12].



surface coverage increased, while at higher concentrations ($>10^{-3}$ mg/ml), adsorption became more difficult as the surface coverage increased.

At steady state, Eq. [10] becomes

$$\Pi_s = \Pi_{\max}[(b + ak_2/k_1)/(1 + ak_2/k_1)]. \quad [13]$$

Under some conditions, values of k_1C and k_2C estimated from Eq. [13] might not be very different from what can be estimated using Eq. [12]. This would be particularly true at high concentrations, at which b is expected to be close to 1 and k_2/k_1 is very small. Accordingly, b can be roughly estimated by substituting k_1C and k_2C obtained from Eq. [8] into Eq. [11]. b -values obtained for nisin and β -casein are listed in Table 7.5.

Table 7.5. b values based on assuming Eq. [13] to be equivalent to Eq. [12] at steady state.

Concentration (mg/ml)	Nisin	β -Casein
1	1.000	1.000
0.5	0.985	0.994
0.1	0.944	0.998
0.01	0.808	0.913
0.001	-	0.360
0.0001		-

- Negative value was obtained

Negative b -values were obtained for the fitted data of each protein at the lowest concentration. This might be due to the shortcomings of the assumptions that allowed for

the estimation of adsorption rates. The areas of state 1 and 2 might not be constant with changing concentration.

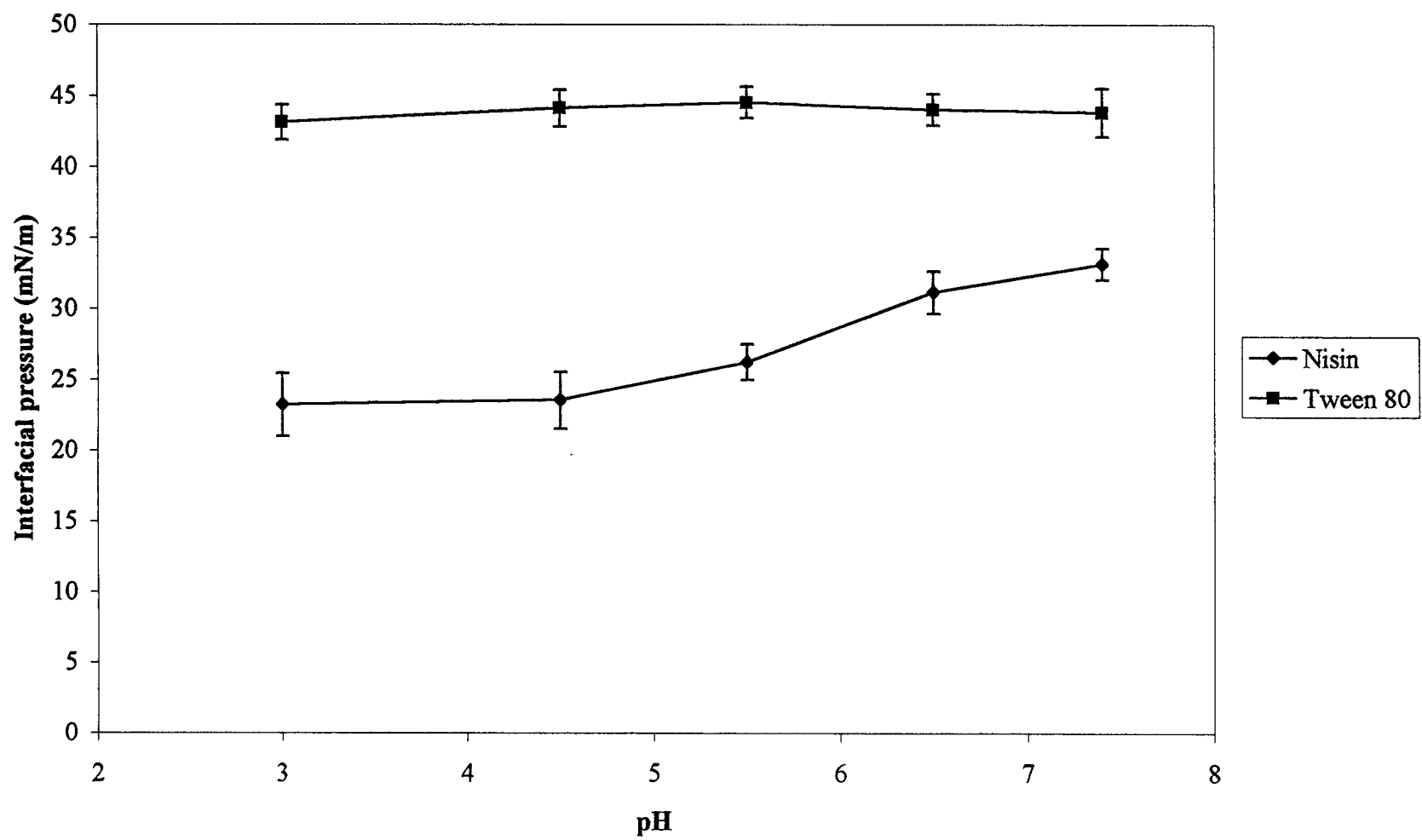
The two states mechanistic model might not work well at very low concentrations, due to the fact that adsorption from highly diluted solution is transport-limited process; i.e., the rate of diffusion to the surface is slower than the rate of protein binding to the surface (24). In addition, protein adsorption at such low concentrations might not result in complete coverage of the interface at steady state. In addition, adsorption rate constants might not be constant during the entire adsorption period; as surface coverage increases, the energy barrier to adsorption may also increase (24).

Modeling of protein adsorption in two states allows for better understanding and quantification of the effect of bulk concentration on conformational changes of proteins at interfaces and more accurate prediction of their behavior when present in a mixture, such as competitive adsorption and desorption. All this is important for better understanding of any process or system that involves contact between some material and protein-containing fluid, such as emulsions, membrane-based separation, adsorption-based recovery of proteins and peptides in downstream processing, and any operation susceptible to bacterial attachment and biofilm formation.

Effect of pH on interfacial pressure of nisin at oil-water interface

Figure 7.11 shows that, compared to Tween[®] 80 (a nonionic surfactant), nisin interfacial activity is reduced as the bulk solution is more acidic. Generally, the greater the reduction in interfacial tension, the stronger the surfactant property of protein. This correlates very well with the functional hydrophobicity (19). Nisin exhibits lower

Figure 7.11. Effect of pH on interfacial pressure.



hydrophobicity, with the reduction of pH. The adsorption of nisin at bacterial cells is pH dependent (25), with a maximum adsorption at pH 6.5, and less than half of that being adsorbed at pH 4.4 (26). Accordingly, the low interfacial activity of nisin in acidic conditions might be due to low adsorbed mass, poor hydrophobic association with the oil phase or both. These results were expected, as it was found that nisin exhibits higher emulsifying activity at neutral than acidic pH (27).

CONCLUSIONS

Nisin, in comparison to β -casein and Tween 80, can adsorb at oil water interface and reduce the interfacial tension significantly. Modeling of the interfacial tension data of nisin and β -casein at an oil water according to a model that allows for adsorption in two dissimilar states, has shown that adsorption at high concentrations occurs in extended form and at low concentrations in compacted form. The model was limited with the assumptions of the same reduction of interfacial tension per unit surface area by the two states, and no effect of diffusion on adsorption, which might be significant at very low concentrations.

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CHAPTER 8

EFFECT OF NISIN ON ERYTHROCYTES, AND DRUG RELEASE FROM OIL-IN-WATER EMULSIONS

ABSTRACT

Nisin is a polypeptide (3510 Da) antibacterial substance produced by the fermentation of a modified milk medium by certain strains of the lactic acid bacterium, *Lactococcus lactis*. It shows antimicrobial activity against a range of Gram positive bacteria, particularly spore formers. The hemolytic effect of nisin on red blood cells was studied. RBCs hemolysis was measured as the increase in UV absorbance as a result of hemoglobin release. Nisin did not hemolyse red blood cells at concentrations less than 0.15 mg/ml, but at higher concentrations hemolysis increased linearly and significantly with increasing concentration. Drug release from oil-in-water emulsions stabilized by nisin was studied and compared to that from emulsions stabilized by Tween[®] 80. It was found that nisin retards drug release from oil-in-water emulsions.

INTRODUCTION

Nisin is a cationic polypeptide secreted by some lactic acid bacteria. It possesses antimicrobial activity against a broad spectrum of Gram positive bacteria and has realized commercial application as a food preservative (1). Nisin is a highly surface active molecule whose adsorptive properties were recognized as early as 1949 when it was noted that nisin in culture remained with *Lactococcus lactis* cells rather than in the culture broth at pH 6 (2). Nisin has been considered to produce the same effect as a cationic, surface-active compound with a detergent effect on the bacterial cell membrane because leakage of UV absorbing material from treated bacterial cells was observed (3).

Nisin is reported to not hemolyse sheep or human erythrocytes (4). These results were expected as the threshold potential required for nisin to induce pore formation in cytoplasmic membranes, at pH 7.5, is 50 mV (5), while the membrane voltage of erythrocytes is -10 mV (4).

Nisin was found to form a gel-like structure at oil-water droplet interfaces (6). As a result, deformed particles of oil-in-water emulsions stabilized by nisin were observed under the microscope. Accordingly, nisin as an emulsifier might affect the release of an emulsified drug species by two mechanisms. The gel structure might retard diffusion across oil droplets as a result of high interfacial viscosity. Irregular shape oil droplets has higher area/volume ratio than spherical ones. Consequently, enhancement of the release rate and extent might be caused by the deformation in oil droplets. Shah et al. (7) investigated the efficiency of various types of polyglycolized glycerides in self-emulsifying drug delivery systems by evaluating the rate and extent of drug release. They found high correlation between average droplet size of an emulsion and drug release rate.

In emulsions, drugs are absorbed mainly into the aqueous phase; the absolute volume of the aqueous phase is critical for poorly oil-soluble drugs ($K < 1$); the amount of drug in the aqueous phase is important for $K > 1$, where K is the oil-in-water partition coefficient of the drug (8). When the initial amount of drug in the aqueous phase is large in comparison with the total amount of drug or when little drug exists in the aqueous phase initially, the rate-limiting step for absorption is drug transfer from oil to water rather than from oil to membrane (8). Emulsions are more effective for lymphatic delivery than aqueous solutions when injected directly into malignant lymphomas. The emulsifier can greatly influence the amount of drug bound to oil droplets as demonstrated oil-in-water emulsions of mitomycin C, where gelatin (an animal protein) gave better results than polysorbate 80. Gelatin microsphere oil-emulsions showed enhanced and sustained delivery of bleomycin in comparison to intravenous and topical injection of aqueous solution (8).

In this study, the effects of nisin on drug release from oil-in-water emulsions and red blood cells hemolysis were studied.

METHODS

Red blood cells hemolysis

Red blood cells hemolysis was done as described previously (4,9). A 0.7% suspension of sheep erythrocytes (SRBC) in phosphate-buffered saline was incubated at 37 °C for 30 min in the presence of various concentrations of nisin or saponin. Erythrocytes were removed by centrifugation and hemolysis was recorded as increasing absorbance at 540 nm.

Drug release studies from oil-in-water emulsions

USP XXII, Dissolution Apparatus (Van-Kel Industries, Inc.) was employed to study the drug release in emulsions. Because it can be easily dissolved in soybean oil and assayed by UV, salfasalazine was selected as a model drug. The formulations that were tested are shown in Table 8.1.

The release of salfasalazine from each formulation was tested in 900 ml distilled water. Alkamuls EL-719 (HLB = 16, polyoxyethylated (40) castor oil , non ionic surfactant from Rhone-Poulenc) was added to dissolution media at concentration of 5% to provide sink condition and permit quantitation of the drug release (7).

Samples were drawn at 5, 10, 15, 30, and 60 min, filtered through 0.2 µm filter and then analyzed by UV at 360 nm. For comparison controls, all experiments were repeated using the surfactant Tween® 80 in place of nisin.

Table 8.1. Formulation of emulsions.

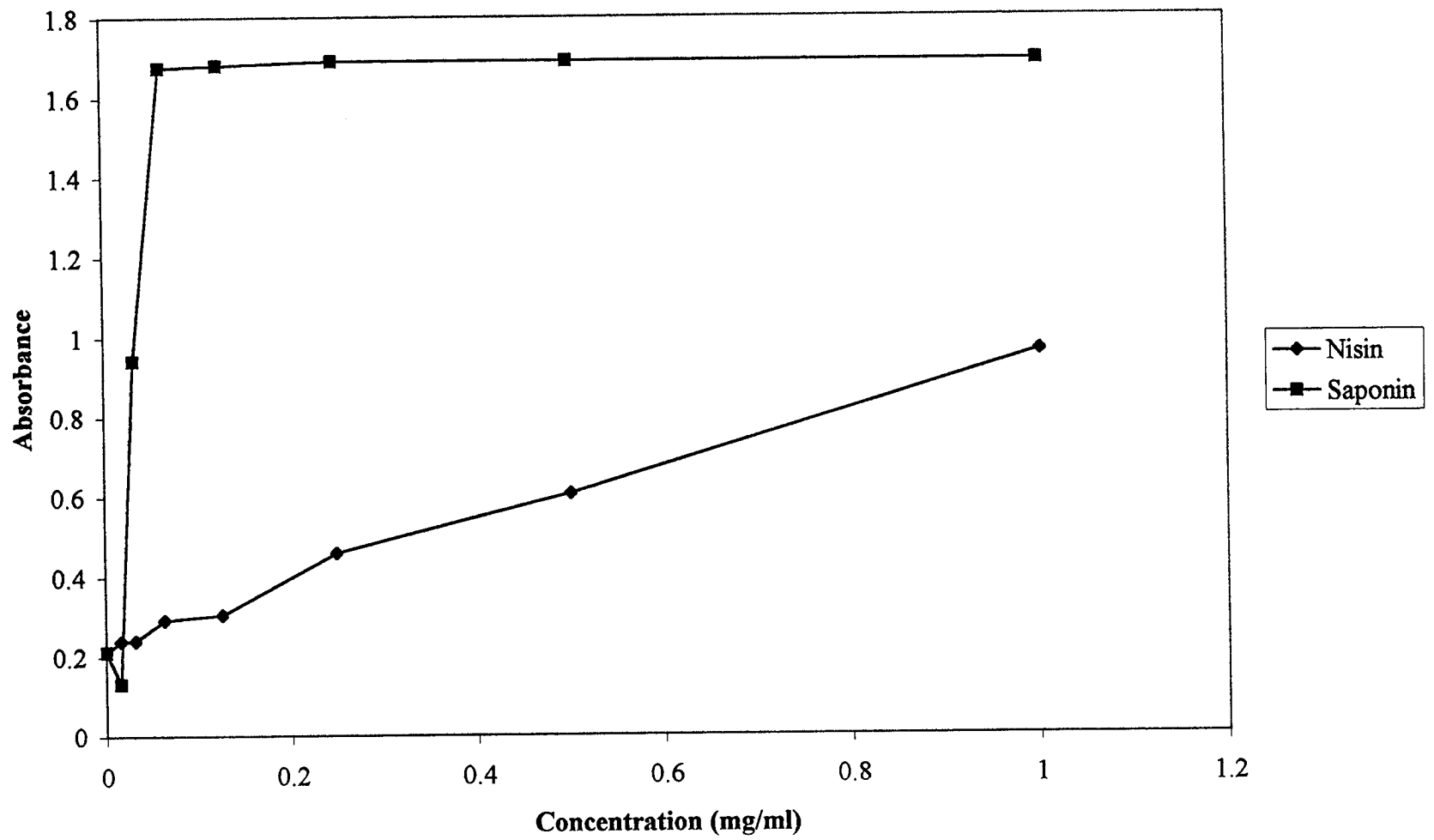
Ingredient	Formulation	
	I (% w/w)	II (% w/w)
Sulfasalazine	4	4
Nisin	0.2	0.4
Soybean oil	40	40
Distilled water	55.8	55.6

RESULTS AND DISCUSSION

Red blood cell hemolysis

Kasschau et al. (9) measured the hemolytic activity of the adult *Schistosoma mansoni* membrane fraction. They measured RBCs hemolysis by assaying the released hemoglobin spectrophotometrically at 540 nm. In this study, hemolysis was measured as the increase in absorbance at 540 nm. Compared to saponin, nisin showed insignificant hemolytic effect on sheep red blood cells (SRBC) at concentrations less than 0.15 mg/ml (Figure 8.1). However, at higher concentrations, SRBC hemolysis was found to increase significantly and linearly with increasing nisin concentration (Figure 8.1). Saponin showed complete hemolysis of the SRBC at concentration of about 0.05 mg/ml. Defined as concentration that hemolyzes 50% of SRBC, the hemolytic titer was calculated to be 0.25 mg/ml for nisin and 0.03 mg/ml for saponin. In a similar study, Kordel and Sahl (4) found that nisin in concentrations up to 1 mM (0.0035 mg/ml) increased the UV

Figure 8.1. Hemolytic effect on suspended red blood cell.

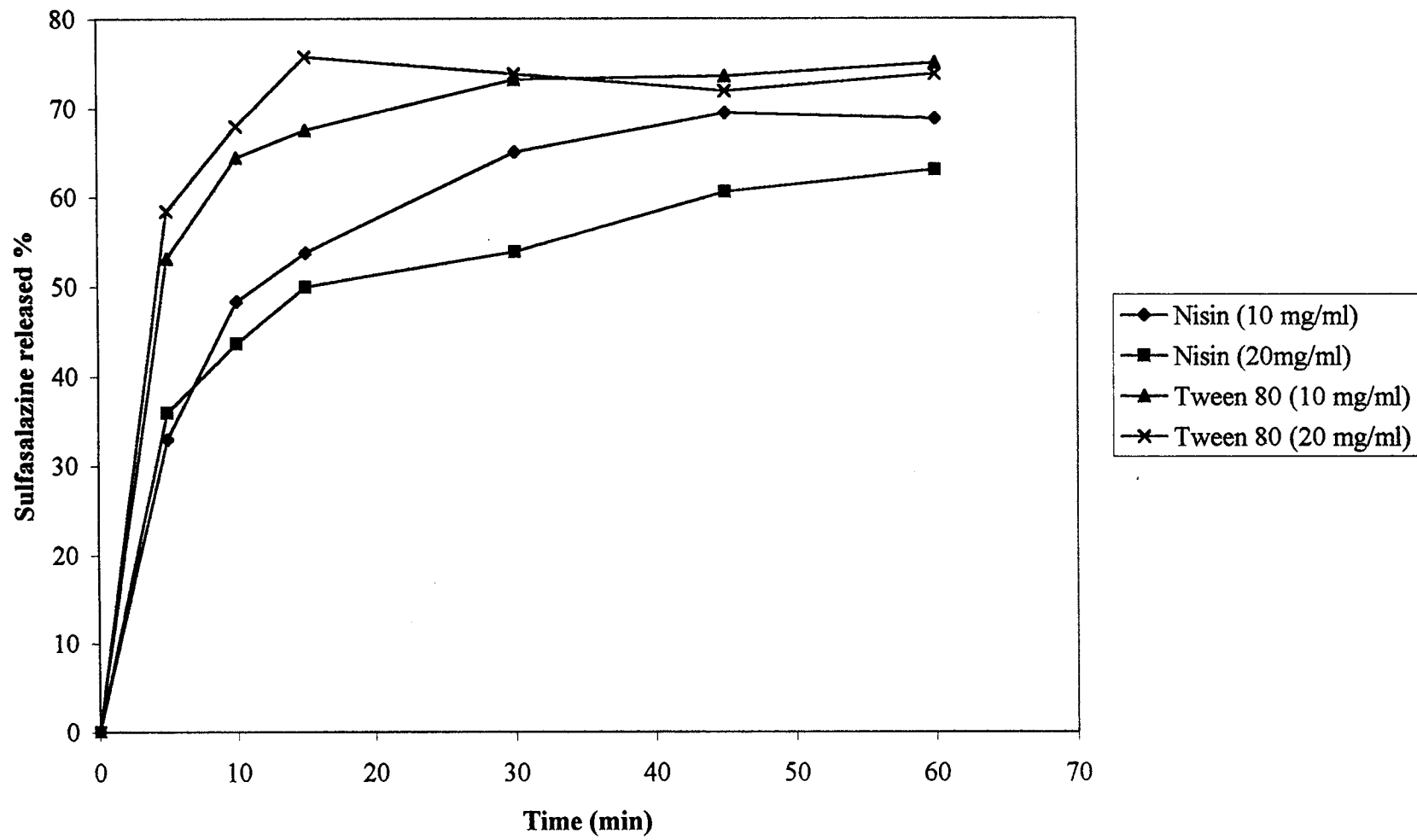


absorbance by only 6 %. In this study, nisin in concentrations up to 0.0313 mg/ml increased the UV absorbance by not more than 12%. Nisin can form pores in cytoplasmic membranes, consequently, nisin might only increase the permeability of RBCs for intracellular substances without total hemolysis. Microscopical examination would be a valuable tool to investigate these effects on RBCs.

Drug release from oil-in-water emulsions

Figure 8.2 shows that the release of sulfasalazine is slower from emulsions stabilized by nisin than from emulsions stabilized by Tween[®] 80. This effect was more profound at higher concentrations. Nisin has been shown to form a gel-like structure at oil-water interface (6). This structure might increase the interfacial viscosity and retard the diffusion of drug from the oil phase into the aqueous phase. It is highly unlikely that these differences are due to differences in particle size, because nisin at the used concentrations produces more stable oil-in-water emulsions than Tween[®] 80 (7).

Figure 8.2. Sulfasalazine release from oil-in-water emulsions.



CONCLUSIONS

Nisin can hemolyze red blood cells, which might be due to its high surface activity and pore formation in the cytoplasmic membrane. Nisin can retard the release of emulsified drug from oil in water emulsion which might be due to gel formation at oil water interface.

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Formulation and *In Vitro-In Vivo* Evaluation of a New Compression-Coated Tablet of Amoxicillin/Clavulanate; and Formulation Potential of the Antimicrobial Peptide Nisin

CONCLUSIONS

Compression-coated tablet can be used to separate active ingredients in the same dosage form. It consists of three layers, and each layer must have good friability and compressibility. Consequently, compression of the outer coat into the hard inner core is challenging. Compression-coated tablet formulation was developed for amoxicillin and clavulanic acid. Amoxicillin in the outer layer was separated from clavulanic acid in the core by inactive ingredients in the middle layer. Stability of clavulanic acid in the core was optimized by incorporation of hydrophobic ingredients in the middle layer. Because of the formulation design, the dissolution profiles of amoxicillin and clavulanic acid from the compression-coated tablet were different from those of the conventional single layer tablet.

The pharmacokinetic parameters of a new compression-coated tablet formulation of amoxicillin/clavulanate and Augmentin[®], a reference product, were evaluated employing a randomized balanced cross over study. Preliminary statistical pharmacokinetic analysis was done for 8 subjects. Data were analyzed by a two one-sided t-test. The two formulations were bioequivalent for amoxicillin for all the pharmacokinetic parameters. However, the two formulations were not bioequivalent for clavulanic acid for AUC and C-max, which might be due to the small number of subjects used in the study.

Nisin is a highly surface active cationic polypeptide with antimicrobial activity. It can withstand activity loss during thermal processing, and exposure to acidic environments. These characteristics and others, such as non-toxicity and surface activity, make its potential as an antimicrobial emulsifier attractive in food and pharmaceutical emulsions. The emulsifying activity of nisin was evaluated using conductivity measurements. The results have shown that nisin has high ability to stabilize oil in water emulsions in comparison to Tween 80 and β -casein. The emulsifying activity of nisin is highly concentration and pH dependent. It increases sharply with increasing concentration and pH.

The interfacial tension kinetics of nisin and β -casein were evaluated at n-hexadecane-water interface using DuNoüy tensiometer. In order to account for the effect of conformational change during adsorption, the interfacial tension kinetic data of each protein were analyzed with reference to a two state mechanism that allows for protein to adsorb in structurally dissimilar forms. The model has shown that adsorption at high concentrations occurs in extended form and at low concentrations in compacted form. The model was limited with the assumptions of the same reduction of interfacial tension per unit surface area by the two states, and no effect of diffusion on adsorption, which might be significant at very low concentrations.

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