EVALUATION OF CALCIUM ALGINATE BEADS AS A PROLONGED RELEASE DELIVERY SYSTEM FOR AN ORALLY ACTIVE IRON CHELATOR

BY

JASON A. ZASTRE

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE
IN PHARMACY

Faculty of Pharmacy
University of Manitoba
Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

Cooley's anemia or β-thalassemia major affects over 100 000 newborns yearly. Treatment involves blood transfusions every 2-4 weeks for the rest of their life. This therapy leads to extreme iron overload resulting in severe life threatening complications. Current therapy for iron overload requires s.c. or i.v. infusions of an iron chelator for 8-12 h per day up to six times a week, causing many patients to be noncompliant. A novel orally active iron chelator 1,2-Dimethyl-3-Hydroxypyrid-4-one (DMHP) has been developed, but the short half life means dosing several times per day. Encapsulation of this novel orally active iron chelator utilizing calcium alginate beads to facilitate a prolonged release was investigated. Beads were produced by dripping a sodium alginate solution containing 15 mg/mL DMHP into a curing solution of 4% $CaCl_2$, and allowed to cure for 15, 30, 45, and 60 min. Beads were then tray dried at 40 and 60°C for 24 h. Dissolution tests were done using the basket method over 6 h. in pH=2.0 HCl-KCl buffer and pH=7.4 phosphate buffer. Beads tested in pH=2.0 buffer did not swell or rehydrate. Complete release of DMHP was obtained within 45-60 min facilitated by diffusion of DMHP through the pores and cracks of the bead structure. Release in pH=7.4 media was prolonged due to swelling and slow erosion of the beads. An immediate release phase was evident within the first 15 min. followed by a sigmodial shaped phase. Additives such as HPMC, propylene glycol, and pectin were included to control the rapid release in pH=2.0 medium, but none delayed the release rate. In pH 7.4 buffer, calcium alginate-pectin beads conformed to the zero order release model (R^2 = 0.9788 – 0.9945). Therefore the beads would have to be enteric coated to prevent quick release in the acidic environment of the stomach and to allow a prolonged release in the intestine.

ACKNOWLEDGMENTS

First and foremost I would like to express my deepest love and thanks to my wife Bobbi-lynn and my daughter Halie for their extreme patience. I know this took a long time, but your sacrifice and understanding has allowed me to persevere. If it wasn't for you, Bobbi, I feel that this would never have been finished. As for my beautiful daughter, Halie, watching you grow and learn so quickly for the last year and a half has inspired me to continue the struggle and finally finish!

I would also like to thank Dr. Keith Simons for his guidance and valuable comments on this manuscript. Your commitment towards my completion of this project will not be forgotten.

To all the other students in the lab and the staff at the Faculty of Pharmacy who have encouraged and contributed towards my education, I thank you. Furthermore, I would like to thank the reviewers for taking the time to evaluate this work.

Finally I want to thank Merck Frosst and the Manitoba Health Research Council for their financial support. Without them none of this could have been possible.

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INTRODUCTION

The presentation of iron overload in a patient can be acute as in accidental poisoning, or chronic such as hemochromatosis, hemosiderosis, and thalassemia. Iron overload is primarily treated by control of symptoms and removal of excess iron by the use of chelating agents. These agents present unique problems in dose administration and compliance. The major goal of this research is to design and evaluate several potential prolonged release dosage forms of an iron chelator to improve chelation treatment.

1.1. THALASSEMIA

Thalassemia is a genetic disorder characterized by a defect in the synthesis of one or more hemoglobin subunits. It has been estimated that over 100 million persons are carriers of the defective gene, and that 100 000 babies are born each year with some form of thalassemia.[1] This disease exists primarily in people of Mediterranean background and from the Middle east, the Indian subcontinent, and southeast Asia. Thalassemia can be divided into two main categories: 1) α-thalassemia where patients have impaired

production of α-globulin subunits and 2) β-thalassemia where there is a decrease in the production of β-globulin subunits.

β-Thalassemia, characterized by a decrease in the production rate of hemoglobin β-chains, causes polymerization of the α-chains to form tetramers of decreased solubility, resulting in the formation of insoluble aggregates within the red blood cell.[2] This brings about a shortened life span for the red blood cell and intermedullary erythroid destruction, resulting in ineffective erythropoiesis and peripheral hemolysis.[2] Since each parent contributes one β-chain gene, resulting offspring can be homozygous and inherit both defective genes, or heterozygous having only one defective gene. For the heterozygote, also known as β-thalassemia minor, illness presents as mild anemia with microcystosis and hypochromia.[2,3] This condition is not considered life threatening or clinically significant and is usually not treated.

Cooley's anemia or β-thalassemia major is when the offspring inherits both defective genes for the β-chain (homozygotes). These patients exhibit severe anemia usually appearing within 4 to 6 months of life. Clinical manifestations are marked wasting and appearance of malnutrition, skeletal abnormalities from expansion of erythroid marrow, enlargement of the malar bones yielding the characteristic "chipmunk" faces, cardiomegaly, spleenomegaly, and hepatomegaly.[2,3,7] Patients surviving to adolescence usually have the development of secondary sex characteristics delayed.[2,7] The life expectancy of these patients is relatively short and very few reach adulthood.

Severely affected patients depend on blood transfusions every 2-4 weeks on a chronic basis, treatment that usually extends life to early adulthood. However, this leads to other complications in these patients. Each unit of blood provides approximately 225

mg of iron, and since there is no mechanism for iron excretion and erythropoiesis is not optimal, the iron accumulates in their bodies. In the normal healthy individual, the body usually contains 4-5 g of iron with an average daily loss of 1-3 mg that is replaced by an equivalent amount. Transfused β-thalassemia patients can accumulate over 50-70 g of iron throughout a 10 year period.[1] The patients usually die in early adulthood from the complications of iron overload, primarily cardiac, hepatic, and endocrine abnormalities.

1.2. TREATMENT OF IRON OVERLOAD

The main treatment of iron overload in transfusion dependent anemias such as thalassemia is the use of iron chelating agents. The only drug clinically used currently, and for the last 20 years, is Desferrioxamine, marketed under the trade name Desferal by Ciba Geigy (Figure 1). Desferrioxamine is a fungal siderophore secreted by microorganisms to scavenge for iron in their environment and is very selective for iron(III) with very low affinities for other elements like calcium, magnesium, copper, and zinc.[1] The main disadvantage of desferrioxamine is that it is not appreciably absorbed when given orally and therefore requires subcutaneous (s.c.) or intravenous (i.v.) administration. It has a very short biological half life of 5-10 min., and when given i.v. or s.c. is excreted in the urine as the free drug, the iron complex (ferrioxamine), and various metabolites. These rapid elimination properties complicate the use of desferrioxamine in chronically iron overloaded patients.

Since desferrioxamine has a very short half life and not orally bioavailable, it is administered by either s.c. or i.v. infusion for a period of 8-12 hours per day up to six times per week.[4,5] Although this route of drug administration is very successful for

reducing the iron load for these patients. most have poor compliance with this treatment and subsequently become further overloaded with iron.

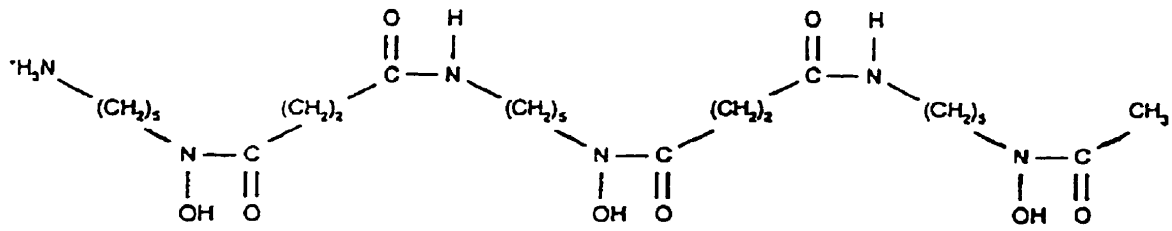


Figure 1: Structure of desferrioxamine. Ref. (24)

The major reasons for noncompliance is pain, reduction in freedom, disturbed sleep, local swelling at the infusion site, and boredom.[6] Socioeconomic factors also play a role in noncompliance, as the treatment is very expensive and very few can afford it. Other concerns of desferrioxamine therapy come from its toxic adverse effects. Chronic administration yields visual, auditory, and neurological toxicities while hypotension, renal insufficiency, growth retardation, and opportunistic infections have also been reported.[11-14, 21,22]

The high degree of non-compliance and the increased incidence of toxicities with desferrioxamine therapy have stimulated the search for possible alternative drugs as potential replacements. Possible approaches include:

1) Modification of desferrioxamine structure.
2) Use of a suitable vehicle to enhance oral absorption.
3) Alternative routes of administration.
4) Development of new orally active iron chelators.

The use of desferrioxamine suppositories have been used to replace s.c. infusions, but resulting iron excretion is only 10% of that achieved with s.c. infusions.[8] Reduced iron excretion is similar following oral administration of desferrioxamine.[9,10] Another novel method is to administer desferrioxamine using liposomes, but only temporary depletion in iron levels could be achieved.[29]

Development of new iron chelators should yield compounds which are inexpensive, promote compliance, and remove excess iron from the body, while exhibiting no more toxicity than current therapy. Most attempts at altering desferrioxamine structurally and in its release have failed. However the development of a new set of orally active iron chelators has received much attention and has instilled new hope for the treatment of iron overload.

1.2.1. THE HYDROXYPYRIDINONES

There are several criteria that need to be addressed for the development of an alternative drug for iron chelation. Firstly, for selective iron chelation the chemical structure of any new compound should contain a hydroxamate and/or catachol functional group. Unfortunately these groups also reduce the clinical usefulness since hydroxamates are susceptible to hydrolysis in acid environments like the stomach and catachols are easily oxidized in the intestine as well as being poorly absorbed.[1] Other chelating compounds contain polyaminocarboxylic acids such as EDTA which will bind iron, but

are very non-specific and can also remove other divalent ions such as calcium, zinc, and magnesium. Secondly, for effective absorption through the GI tract the molecule and the resulting iron complexes should remain as a neutral species throughout the entire pH range of the GI tract. A thorough evaluation of potential compounds that are acid stable, orally absorbable, and have efficient specific iron chelating properties has lead to the development of a new class of iron chelators, the hydroxypyridinones.

1.2.1.1. 1,2-DIMETHYL-3-HYDROXYPYRID-4-ONE

Many derivatives of the hydroxypyridinones have been tested for iron chelating properties *in vitro* and *in vivo* in mice and rabbits.[15-18] The most promising compound is 1,2-dimethyl-3-hydroxypyrid-4-one also known as L1, CP20, DMHP, or Deferiprone (Figure 2). Successful preliminary animal studies have resulted in encouraging clinical trials for iron chelation therapy following oral administration.[19, 20]

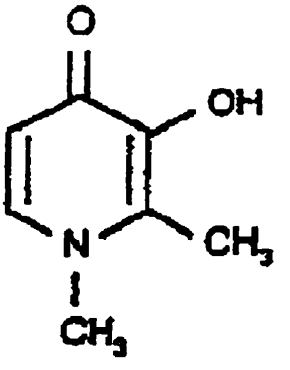


Figure 2: Structure of 1,2-dimethyl-3-hydroxypyrid-4-one. Ref. (24).

Studies comparing DMHP to desferrioxamine for efficient iron removal in overloaded patients showed both compounds to be comparable at equivalent doses.[23, 28] The dose used for DMHP ranges from 50-125 mg/kg/day orally compared to desferrioxamine doses of 50-100 mg/kg/day as a 12 h. infusion. The LD_{50} of DMHP is >1 g/kg in various animal species. Adverse effects reported in humans include reversible agranulocytosis, arthropathy, gastric intolerance, and zinc deficiency.[24-27]

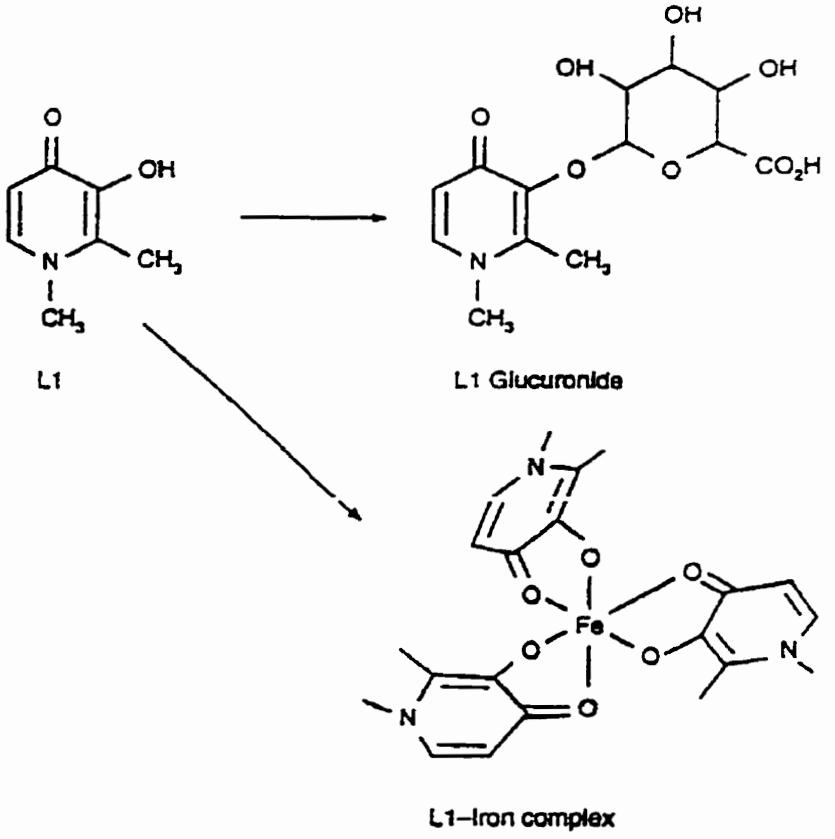
1.2.1.1.1. PHYSIOCOCHEMICAL PROPERTIES

DMHP (mw=139.15) crystallizes as white orthrombic crystals. The melting point is 266-268^{0}C. Solubility in water at 24^{0}C is 16-18 mg/mL.[30] It is very stable at room temperature in both acidic and basic solutions.[30] The nitrogen in the pyridine ring exhibits a pKa_1=3.3 and the 3-hydroxy group possesses a pKa_2=9.7.[31] Evaluation of ion specificity shows that DMHP exhibits a stronger affinity for iron(III) compared to other ions like calcium, magnesium, and manganese, but has moderate affinities for aluminum and copper.[37] Complexation with iron over the pH range of 6-10 forms neutral species of purple to orange color with a 3 DMHP: 1 Fe stoichiometry (Figure 3).[31] This complex has a stability constant ($\log\beta_3$) of 36.34 compared to desferrioxamine of 30.60 with a λ_{max} = 460 at pH=7.3.[31,32] The partition coefficient (K_{part}) of free DMHP is 0.21 and of the iron complex is 0.0009.[33]

1.2.1.1.2. PHARMACOKINETIC PROPERTIES

The absorption half life of DMHP ranges from 7-22 min., suggesting a rapid absorption from the stomach or upper GI tract.[34, 35] The t_{max} is approximately 1 h.

followed by elimination primarily via the kidneys and a $t_{1/2elim}$ ranging from 74.3-159.6 min.[34,35,36] The majority of the drug is eliminated as free DMHP, plus the glucuronide conjugate, and the iron-DMHP complex (Figure 3).[34] The relatively short half-life of DMHP indicates it will need to be administered several times daily to maintain therapeutic blood levels, or require the development of a modified release drug delivery system.



L1–Iron complex

Figure 3: Structures of DMHP, DMHP-glucuronide, and DMHP-iron complex. Ref. (24).

1.3. MODIFIED RELEASE DOSAGE FORMS (MRDF)

To date there have been numerous terms describing specialized release dosage forms. These include: Continuous action, Slow release, Controlled release, Sustained release, Time release, Slow acting, Long acting, Prolonged release, Retarded release. Delayed release. Time coat, and others. Many of these terms were developed by drug manufacturers as marketing tools to help distinguish their products. Collectively they describe a drug delivery system that modifies the rate of release of a drug from its dosage form.

In 1985 the USP/NF adopted the term "Modified release dosage form" (MRDF) to avoid confusion between the different manufacturers descriptive terms. It is defined as "one for which the drug release characteristics of time course and/or location are chosen to accomplish therapeutic or convenience objectives not offered by conventional dosage forms such as solutions, ointments, or promptly dissolving dosage forms."[38] This definition attempts to link together the subtle differences between the various specialized release systems. The USP/NF also recognizes two distinct types of modified release dosage forms, delayed release and extended release.

A delayed release dosage form is defined as one that releases the drug substance at a time other than immediately after administration.[38] An example is an enteric coated tablet which will not release the medication in the acidic environment of the stomach but allow its release in the less acidic environment of the intestines.

An extended release dosage form is one that allows at least a two-fold decrease in dosage frequency compared to the conventional immediate release form.[38] Extended release systems can be subdivided into two types, prolonged/sustained release or

controlled release. Prolonged or sustained release dosage forms are designed to release the drug substance slowly over an extended period of time (Figure 4). Controlled release systems imply some predictability and reproducibility of drug release. Initially a loading dose is released to obtain a rapidly achieved therapeutic blood level, followed by a slower, more constant release of the drug where the amount eliminated from the body is being constantly replaced (Figure 4).

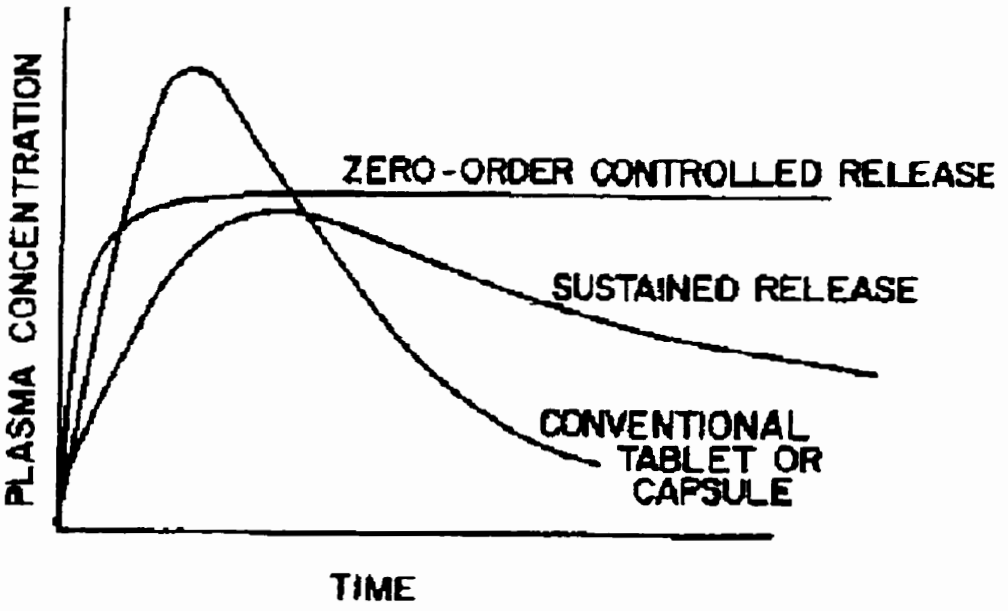


Figure 4: Comparison of the drug-plasma concentration vs. time curve of a conventional tablet, a sustained/prolonged release system, and a controlled release dosage form. Ref. (39).

1.3.1. OBJECTIVES OF MODIFIED RELEASE DOSAGE FORMS

A conventional dosage form is designed to disintegrate immediately upon contact with GI fluid, facilitating immediate release of the medication(s) it contains. This results in quick dissolution, followed by absorption of the compound into the blood. For these types of systems, the rate of drug entry into the blood is not governed by the dosage form but rather by the physiocochemical properties of the drug and the physiological parameters at the absorption site. The administration of several doses results in a "saw-tooth" pattern for the blood-drug concentration curve (Figure 5). These fluctuations can lead to toxic and/or subtherapeutic levels in the blood, resulting in unwanted side effects and/or reduced pharmacological response. This is not ideal for drugs which have a very narrow therapeutic ranges.

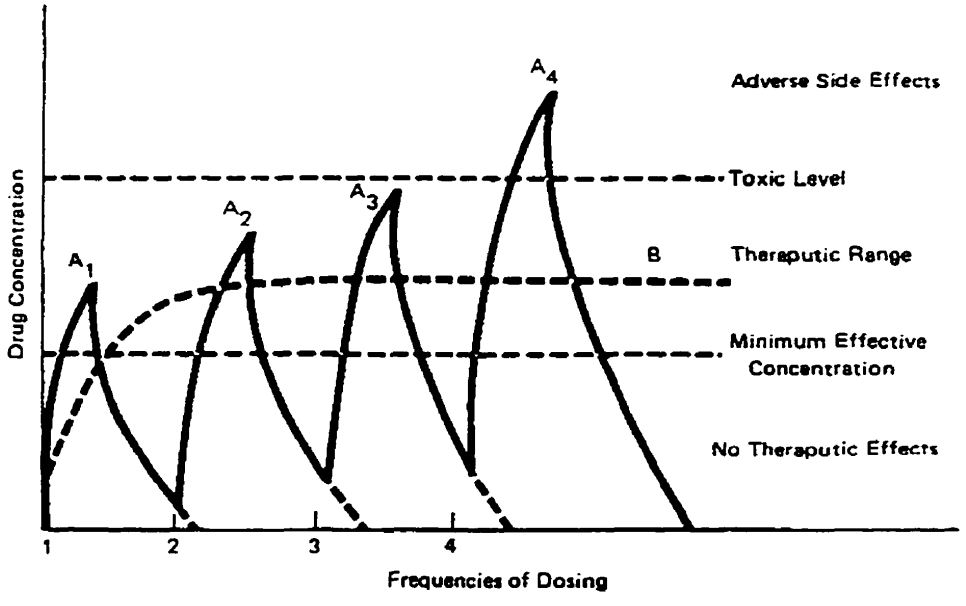


Figure 5: Hypothetical blood drug concentrations resulting from (A) conventional tablets and (B) controlled release tablets. Ref. (40)

The objectives of a modified release system is to alter the pharmacokinetics and pharmacodynamics of a drug in order to minimize drug adverse effects from fluctuations in blood concentrations and provide a prolonged therapeutic effect (Figure 5). In order to achieve this, a system should release the drug substance at a constant rate approximating zero order kinetics so that at steady state, the rate of drug released should equal the rate eliminated from the body (rate in = rate out). For this type of drug delivery system, the dosage forms design controls the rate at which drug appears in the blood.

1.3.2. ADVANTAGES AND DISADVANTAGES

The design of modified release dosage forms holds many advantages over conventional dosage form counterparts. The main advantage is a decrease in dosing frequency. The extended-release nature of these systems allows drugs to be administered once or twice a day and still maintain therapeutic blood concentrations, as compared to the conventional forms that are given three to four times a day. Reduced frequency of dose administration promotes patient convenience and compliance. Modified release dosage forms can also reduce unwanted side effects resulting from fluctuating plasma concentrations and prevents the drug dropping to subtherapeutic levels by maintaining a constant rate of drug release. A once-daily treatment with a modified release dosage form can be less expensive then 3-4 treatments of a conventional form, resulting in better drug utilization and patient compliance.

One major disadvantage of modified release dosage forms is the higher cost of production relative to the conventional dosage form. This is most likely due to the research and money invested in developing such a dosage form. In a poorly formulated

MRDF product there is also the possibility of "dose dumping" of a larger dose so a potentially toxic single dose of the drug substance may be released. Conversely the dosage form may fail to release the drug properly leading to inadequate therapeutic concentrations. Another concern is the physical size of these dosage forms. Some systems are much larger then conventional forms because they contain the equivalent of several conventional tablet doses. Difficulty of ingestion becomes the major complaint from patients, especially geriatric patients.

If the MRDF causes acute intoxication in the patient by "dose dumping" or accidental overdose, it is more difficult to remove the drug than from a conventional dosage form. This is due to the fact that drug is usually released continually throughout the entire length of the GI tract. Treatments such as gastric lavage or forced emesis may not effectively expel the modified release drug dosage form. Fine adjustment of dosage regimens is usually difficult with most systems as they cannot be halved, chewed, or crushed. The major disadvantage of the MRDF is the poor *in vivo/in vitro* correlation. A good modified release profile *in vitro* may not be elicited *in vivo* because of the many biological variances in human or animal subject's sites of absorption.

1.3.3. LIMITATIONS OF DRUG SELECTION

When selecting a drug to be formulated in a modified release dosage form, the physiocochemical characteristics must be considered to ensure the suitability of the formulation.

The drug itself should be rapidly and efficiently absorbed from the GI tract. Once steady state is achieved, the small amount continually released to maintain plasma levels

will need to be rapidly and completely absorbed to reduce any fluctuations in the plasma concentration. A drug with a narrow therapeutic range would also be a prime candidate, since a modified release system can be specifically tailored to release the drug to maintain a relatively constant therapeutic range and reduce fluctuations in plasma concentrations achieved by repeated administration of conventional doses.

Drugs with elimination half lives ($t_{1/2}$) shorter than 2 hours, are usually not selected because of the large amount of drug needed to formulate them. This results in very large dosage forms that can be difficult to swallow. Drugs with half lives longer than 8 hours are not chosen because they can potential accumulate during sustained and constant release. Such drugs are sufficiently sustained in the body when delivered by a conventional dosage form. Drug's which have therapeutic effects independent of plasma concentration are not chosen (e.g. tricyclic antidepressants) as there is no need to maintain a consistent plasma concentration. A final limitation of a candidate drug is the phenomenon of receptor tolerance. Formulating this type of drug in a modified release preparation can elicit faster tolerance effects because the receptors are constantly being exposed to it. Sustained release nitroglycerin is an example of a drug that falls into this category, however some exceptions to this rule do exist.

1.3.4. BIOLOGICAL CONSIDERATIONS

Initially the MRDF is tested using *in vitro* methods, involving mainly dissolution studies in appropriate media. What may seem like a suitable release profile for a modified release dosage form *in vitro* may not necessarily be maintained when tested *in vivo*. This is because of the inter and intra-variability between subjects used to test the

system *in vivo*. The main source of variability for orally administered systems comes from the physiology of the gastrointestinal tract (GI tract). The GI tract can be divided into four major sections that have varying effects on drug bioavailability: the stomach, small intestine, large intestine and the colon or rectum (Figure 6).

Following oral administration of the dosage form, the first organ the MRDF encounters is the stomach. The stomach is involved in the mixing and grinding of food and the secretion of acid and enzymes to help aid in digestion. In the presence of food the stomach undergoes the digestive or fed phase where food particles less than 1 mm are emptied via the pyloric sphincter into the duodenum.[41, 42] This phase can last up to 8 h. with meals of high solid content or finish in less than 60 min. with a liquid diet.[41]

When there is no food present, the stomach undergoes the interdigestive phase or fasting state. In this cycle the stomach undergoes a series of sporadic contractions finally ending with what is called the "housekeeper" contraction which removes all remaining material in the stomach into the small intestines.[41] This cycle of contractions repeats itself every 90-120 min.[41] During these cycles there are secretions which change the environmental pH. The pH of the fasting stomach can range from 1-3, while in the presence of food the pH can rise as high as 3-5. Significant variation can occur in the drug delivery systems residence time in the stomach depending on the presence or absence of food. For example, sustained release ibuprofen tablets can reside in the stomach for up to 11 h. when taken after food, or be eliminated within 35 min. in the fasted subject.[43, 44] Design strategies focus on the structure of the delivery system, as SR granules may leave the stomach faster and more uniformly than large intact erodible or

leaching type tablets. The chemical stability of the drug should also be considered as it can be exposed to the acidic environment for varying periods of time.

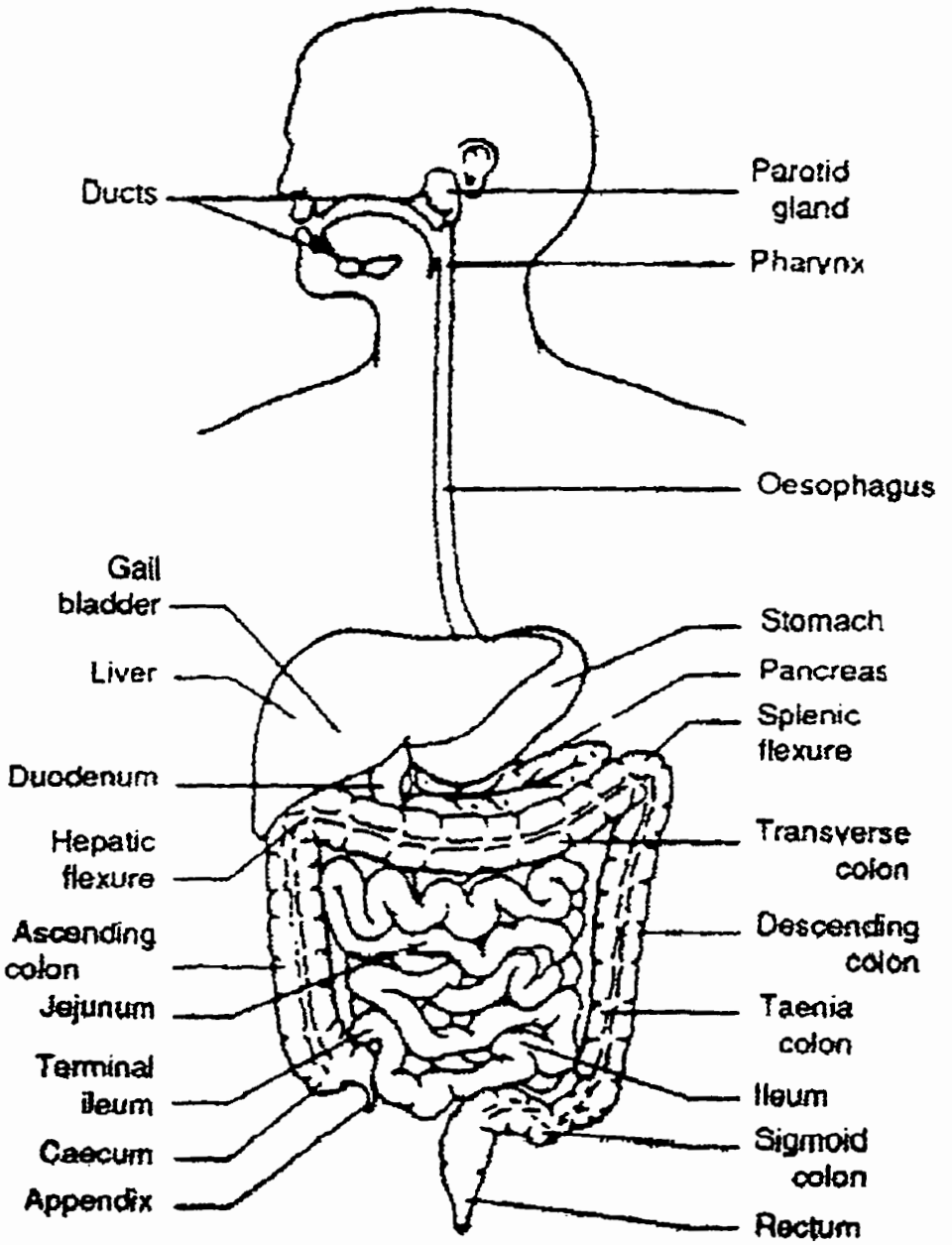


Figure 6: Schematic representation of the gastrointestinal tract. Ref. (41)

Time of passage into and through the small intestine is the most variable parameter between subjects. Here the pH can rise to around 6 or 7 due to the pancreatic secretion of bicarbonate. In adults, the length of the intestine ranges from 10 to 14 feet and consists of three main parts: the duodenum, jejunum, and ileum (Figure 6). The surface of the small intestine is composed of villi that are further capped by microvilli, which greatly increase the surface area for absorption. The density of the villi decreases from the ileum through the intestine to the ileo-cecal junction. The transit time for liquid and solids is ~3-4 hours in the fasting state and ~6-10 hours in the fed state.[11] Therefore depending on the release characteristics of the system, the intestinal transit time may be too short in the fasted state to allow sufficient drug release. For some drugs, absorption takes place only at specific areas of the small intestine, so if intestinal transit time is too fast, efficient drug absorption may be prevented.

Finally the MRDF is exposed to the large intestine in its transit through the GI tract. Here the pH is ~7-8 and MRDF transport is very slow due to decreased fluid in the large intestine. When compared to the small intestine, which is relatively sterile, the large intestine is a huge reservoir of microbial activity. The bacteria here have the potential to metabolize the drug and consequently reduce the bioavailability. Poor absorption of the drug in the colon is mainly due to the lack of villi and fluids. If a MRDF does not release all of the drug content during transit through the small intestine, absorption from the large intestine only can result in considerable variation of results. However once the MRDF reaches the rectum, drug absorption increases in efficiency again due to the network of blood vessels in this area, and in some 12-24 hour MRDF systems, significant absorption will still occur here.

1.3.5. TYPES OF MODIFIED RELEASE DOSAGE FORMS

The most common route of drug administration is the oral route, primarily for patient convenience and acceptability. Modified drug delivery design techniques have focused on the oral route, mainly because of an increase in flexibility of design parameters. Most modified release dosage forms given orally are solid although some liquid formulations do exist. The main reason for this is that most system designs rely on modification of the drug's diffusion and dissolution properties to achieve modified release. Liquids permit the drug particles or molecules to be in contact with the potential dissolution medium immediately, which consequently removes the drug from its dosage environment leading to rapid absorption in the GIT.

In general, most MRDF will contain a dose for immediate release to establish a rapid therapeutic blood level followed by a controlled release dose to maintain that level. The many different approaches in developing MRDF systems are listed in Table I.

Table I: Design strategies of modified release systems. Ref. (41).

1. Continuous release systems:
    a) Dissolution control
    b) Diffusion control
    c) Dissolution and diffusion control
    d) Ion-exchange resins
    e) Osmotically controlled devices
    f) Slow-dissolving salts or complexes
    g) pH-independent formulations

2. Delayed transit and continuous systems
    a) Density based systems
    b) Size based systems
    c) Bioadhesive systems

3. Delayed release systems
    a) Intestinal release
    b) Colonic release

1.3.5.1. DISSOLUTION CONTROL

In order for a drug to be absorbed into the blood stream it must first dissolve in the aqueous fluids of the GI tract. The process of dissolution is governed by the pH of the environment, the pKa of the drug, the surface area of the drug particles. and various other physiocochemical properties of the drug such as polymorphic form. salt form etc. A diffusion layer model developed by Noyes and Whitney in 1897 (Figure 7) describes the rate at which a solid dissolves in a solvent. This model is described by equation (1)

$$\frac{dC}{dT} = \frac{DS}{Vh}(C_s - C) \qquad (1)$$

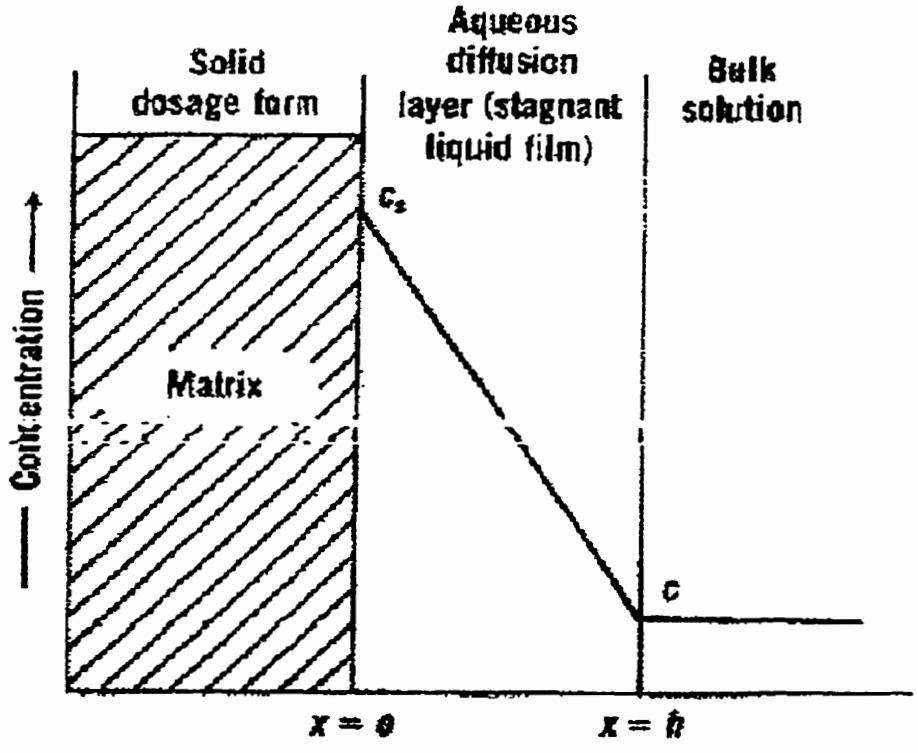


Figure 7: Diffusion layer model. Ref. (57)

Where

$\frac{dC}{dT}$ = dissolution rate
C = concentration of solute at time T
T = time
D = diffusion coefficient of the solute in the solvent
S = surface area of the exposed solid
V = volume of solvent
h = thickness of the diffusion layer
C_S = solubility of the solid

It is assumed that an aqueous diffusion layer or stagnant layer exists between the solid and the solvent of thickness h. This layer represents a stationary layer of solvent through which the solute molecules diffuse, driven by a concentration gradient from C_S to C. At x = h, the solute molecules mix with the bulk solvent and form a uniform concentration, C. At x = 0, the drug in the solid is in equilibrium with a saturated drug concentration in the diffusion layer. The gradient across the diffusion layer is linear.

When C is much less than C_S, the system is said to be in *sink conditions* and the equation can be rewritten as:

$$\frac{dC}{dT} = \frac{DSC_s}{Vh} \qquad (2)$$

In equation (1) and (2), it is assumed that S and h are constant, but this is not always the case. The thickness of the diffusion layer can be reduced by agitation thus increasing the dissolution rate. Also, the surface area of the solid can change as it dissolves. Although these equations may not simulate actual dissolution conditions, they do provide valuable information of ways to alter the dissolution character of a MRDF in order to modify the drug release.

One of the most common techniques for modifying a drug's release is to utilize dissolution as the rate limiting step. Drugs with a slow dissolution rate are inherently sustained because of their intrinsic low aqueous solubility, e.g. digoxin. For compounds with high aqueous solubility, a decrease in the dissolution rate can be achieved by three different methods.

1. Derivatize the drug, form a salt or complex.
2. Incorporate the drug in a matrix that slowly erodes.
3. Encapsulate or coat drug particles with a poorly soluble material

The formation of a slightly soluble salt form of a drug can reduce it's dissolution in the GI tract fluids in order to delay it's release. Phenylephrine as the tannate salt provides constant blood levels for 12 h. after a single oral administration.[45] Coprecipitation of drugs is an example of complexation of a drug to prolong its release. Thioridazine-pectin coprecipatates yielded much slower dissolution rates than that of physical mixtures of drug and lactose suggesting an application in sustained release principles for thioridazine.[46]

Stagnant diffusion layer control assumes that the dissolution process is diffusion layer controlled. Therefore, the rate of diffusion through an unstirred water layer on the solid surface relative to the bulk solution is rate limiting, and thus an increase in the stagnant layer prolongs the dissolution rate of the drug. Such a system can be described by Fick's first law, where the flux, J, is given by:

$$J = -D\left(\frac{dc}{dx}\right) \tag{3}$$

Where

D = Diffusion coefficient

$\frac{dc}{dx}$ = Concentration from the solid surface to the bulk solution side

The material flow rate through a unit area A from a dosage form can be defined as:

$$J = \left(\frac{1}{A}\right)\frac{dm}{dt} \tag{4}$$

Where

A= the effective area available for diffusion.

If the concentration gradient is linear and the thickness of the diffusion layer is h, then

$$\frac{dc}{dx} = \frac{(C_s - C_b)}{h} \tag{5}$$

Where

C_b = concentration of the bulk solution
C_s = concentration at the solid surface
h = diffusion layer thickness

Combining the above equations gives

$$\frac{dm}{dt} = -\frac{DA}{h}(C_s - C_b) = kA(C_s - C_b) \tag{6}$$

Where k is the dissolution rate constant. A constant dissolution rate is predicted using Equation (6) but requires the surface area, diffusion coefficient, diffusion layer thickness, and concentration difference to be kept constant. As dissolution proceeds, all these parameters may change. For spherical particles, the change in area can be related to the weight of the particle. Under the assumption of sink conditions, equation (6) becomes the cube root dissolution equation

$$W_0^{1/3} - W^{1/3} = k'_D \tag{7}$$

Where

k'_D = the cube root dissolution rate constant
W_0 = the initial weight
W = the weight remaining

Another useful equation for determining the dissolution rates of dosage forms with different geometries is:

$$\frac{M_t}{M} = 1 - \left(\frac{1 - K_0 t}{C_0 a}\right)^n \qquad (8)$$

Where

M_t = amount released at time t
M = total amount released
a = half thickness of dosage form
n = constant shape factor: n = 3 for a sphere, n = 2 for a cylinder, n = 1 for a slab

In matrix dissolution the drug is dispersed in a slowly dissolving carrier and compressed into a tablet form (Figure 8b, page 26). The matrix can be one of several polymeric or wax compounds (Table II, page 25). The rate of drug dissolution is dependent on the rate of penetration of the dissolution fluid into the matrix and/or erosion of the matrix. The porosity of the matrix, the presence of hydrophobic additives, and the wetability of the tablet and particle surface control the rate of penetration of the fluid. The type of polymer or wax used governs erosion rate.

Bioerodible tablets are an example of using dissolution to control modified release of a drug substance. Slow erosion of the tablet surface facilitates the exposure of the drug particles to the dissolution fluid, thus controlling the rate of drug release. The mechanism of biodegradation for the polymers usually involves chemically or enzymatically

catalyzed hydrolysis. Wax matrices dissolve at body temperature, or close to it. The most desirable bioerodible system is one where the drug is fully immobilized in the matrix so that diffusional release is of little consequence and release will be facilitated solely by erosion of the outer surface of the matrix. Therefore depending on the final shape of the dosage form, the release kinetics can be described by Equation 8. The disadvantages of erosion devices is that the duration of release of drug from the device following ingestion is directly proportional to tablet thickness, and unless the surface area remains constant, the rate of drug release will decline as the area decreases. Therefore, a release rate approximating zero-order is difficult to achieve with these devices.

Encapsulated dissolution control involves coating individual particles or granules of drug, called cores, with a slowly dissolving material, called a coat (Figure 8a). The time required for the dissolution of the coat is a function of the thickness and the aqueous solubility of the polymer. Employing coats of different thickness on a portion of the particles in a dosage form can help achieve a prolonged release and or repeat action release of the drug.

The principle technique for encapsulating drugs is microencapsulation. It is now one of the most widely utilized and researched methods for producing modified release dosage forms. It's use has extended into the encapsulation of liquids as well as solids. It can be employed to produce many different types of release mechanisms, such as swelling, erosion, inert-nondisintegrating, porous, or dissolving. A discussion of microencapsulation is included later in this chapter.

Table II: Bioerodible polymers and waxes used for matrix dissolution control. Ref (39+47)

1.Waxes
- Glyerol palmito-stearate
- Beeswax
- Glycowax
- Castor wax
- Carnauba wax
- Glyceryl monostearate
- Stearyl alcohol

2. Polymers
   - A. Polyamides
     - Albumin
     - Poly[(hydroxyalkyl)-L-glutamines]
     - Collagen
     - Poly(L-leucine-co-L-aspartic acid)
     - Polydepsipeptide
     - Poly(β-alanine)
     - Poly(proline-co-glutamic acid)
     - Poly(gelatin-co-lysine esters)
     - Poly(L-glutamic acid-co-γ-ethyl-L-glutamate)
   - B. Polyesters
     - Poly(β-propiolactone)
     - Poly(β-hydroxybutyrate)
     - Poly(lactic acid)
     - Poly(glycolic acid)
     - Poly(ε-caprolactone)
     - Poly(alkelyene oxalates)
     - Polydioxanone
     - Poly(alkelyene diglycolates)
   - C. Polyaminotriazole
   - D. Polydihydropyrans
   - E. Poly(alkyl 2-cyanoacrylates)
   - F. Poly(orthoesters)
   - G. Polyanhydrides
   - H. Chitosan
   - I. Polyurethanes
   - J. Polyacetals

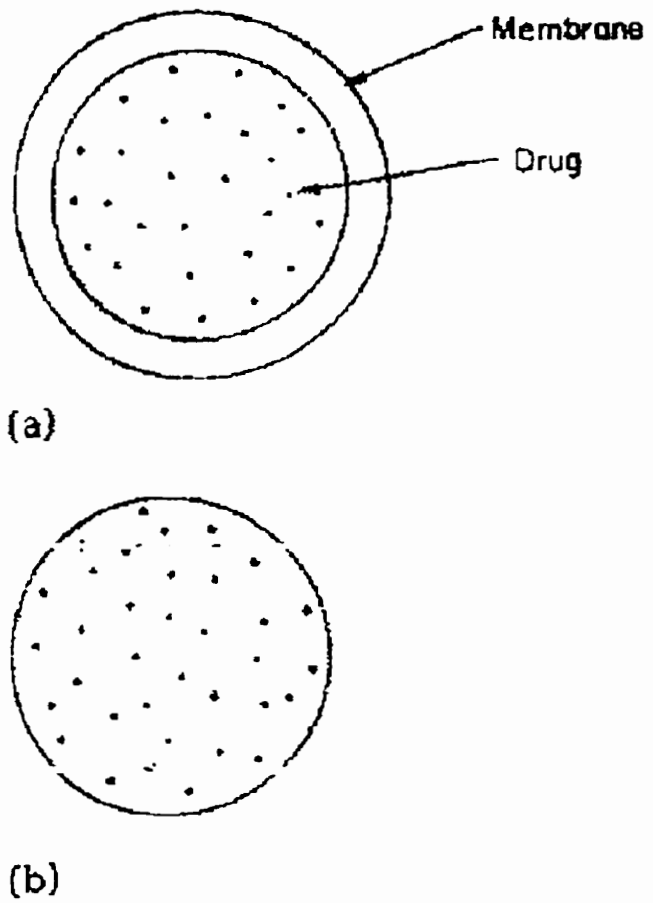


Figure 8: Schematic representation of dissolution controlled drug release a) encapsulated b) matrix. Ref (39)

1.3.5.2. DIFFUSION CONTROL

Diffusion of a drug molecule provides the movement from a zone of high concentration to that of low concentration. Here, the formulator relies on the diffusion of the drug through an inert membrane barrier to control the release rate of a drug. There are two types of diffusion controlled systems:

1. Reservoir devices
2. Matrix devices

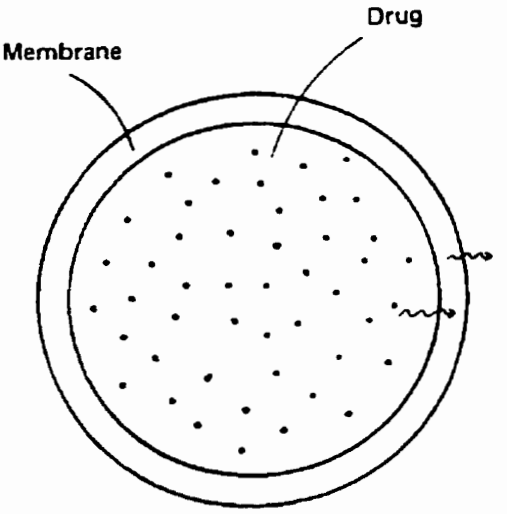
1.3.5.2.1. RESERVOIR DEVICES

A reservoir device can be described as a core of drug (the reservoir) surrounded by a water insoluble polymeric membrane (Figure 9a). Drug release occurs from the partitioning of the drug through the coating membrane to enter the dissolution media. The driving force is the concentration gradient between the core and the dissolution media. The rate of diffusion across the membrane can be described by Fick's first law of diffusion, (Equation 3) which states that the amount of drug passing across a unit area is proportional to the concentration difference across that plane. Assuming that the drug is in equilibrium on either side of the respective membrane surfaces, the concentration just inside the membrane can be related to the concentration in the adjacent region by the following equations (Figure 9B):

$$K = \frac{C_{m(0)}}{C_{(d)}} \quad \text{at } x = 0 \tag{9}$$

$$K = \frac{C_{m(d)}}{C_{(d)}} \quad \text{at } x = d \tag{10}$$

(A)



(B)

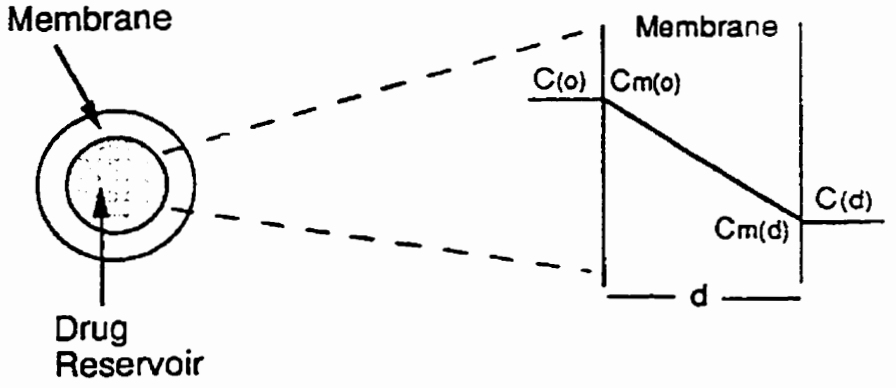


Figure 9: (A) Schematic representation of an encapsulated diffusion control device. Ref (63). (B) Concentration of drug on the inside of the membrane and adjacent regions. Ref (64)

Where

K = partition coefficient (ratio of the drug concentration in the membrane to that in the bathing medium at equilibrium)
C_m = Concentration of drug on the inside surface of the membrane
$C_{m(d)}$ = Concentration of drug on the outside surface of membrane
d = thickness of the diffusion layer

Assuming that K and d are constants, Equation (3) can be integrated to:

$$J = \frac{DK\Delta C}{d} \tag{11}$$

Where ΔC is the concentration difference across the membrane. The drug release will vary depending on the geometry of the system. Therefore Equation (11) can be rewritten depending on the geometry:

Slab:

$$\frac{dM_t}{dt} = \frac{ADK\Delta C}{d} \tag{12}$$

Where

A = the effective surface area of the membrane available for diffusion

Cylinder:

$$\frac{dM_t}{dt} = \frac{2\pi hDK\Delta C}{\ln(r_o/r_i)} \tag{13}$$

Where

h = length of the cylinder
r_o and r_i = outside and inside radius respectively

Sphere:

$$\frac{dM_t}{dt} = 4\pi DK\Delta C\frac{r_o r_i}{r_o - r_i} \tag{14}$$

Where

r_o = outer radius of device
r_i = radius of the inner core

The left side of equations 12 to 14 represents the release rate of the system. In order to maintain a zero order release, the parameters on the right hand side of the equation must remain constant. That includes the diffusional area and path length for a slab, and the inner and outer radius of a cylinder and a sphere. It is somewhat difficult to maintain all of these parameters more or less constant, but zero order release is approximated with these systems. For example, salicylic acid reservoir dosage forms showed varying degrees of zero order release, depending on the thickness of a hydroxypropyl cellulose - polyvinyl acetate coat.[58]

1.3.5.2.2. MATRIX DEVICES

A matrix device consists of a drug homogeneously dispersed throughout an inert or insoluble polymer matrix (Figure 10). The rate of drug release through a planar inert porous or granular matrix has been described by Higuchi.[59, 60] Here, drug is able to pass from the matrix through fluid filled pores, but does not pass through the polymer directly.

$$Q = \left(\frac{D\varepsilon}{\tau(2A - \varepsilon C_s)C_s T} \right)^{1/2} \qquad (15)$$

Where

Q = drug release in grams per unit surface area
D = diffusion coefficient of drug in release medium
ε = porosity of matrix
τ = tortuosity of matrix

C_s = solubility of drug in release medium (g/mL)
A = concentration of drug in tablet (g/mL)
T = time

The equation for a planar system having a homogeneous matrix is:

$$Q = \sqrt{DT(2A - C_s)C_s} \tag{16}$$

Where

D = diffusion coefficient in the matrix

The assumptions made in deriving the Higuchi equations are as follows:

1. A pseudo-steady state is maintained during drug release.
2. A >> C_s. i.e.. excess solute is present.
3. C = 0 in solution at all times (perfect sink).
4. Drug particles are much smaller than matrix particles.
5. The diffusion coefficient remains constant.
6. No interaction occurs between the drug and the matrix.

For the purposes of data treatment, Equation 15 and 16 are often simplified assuming the matrix parameters remain constant:

$$Q = KT^{1/2} \tag{17}$$

A plot of amount of drug release versus the square root of time should be linear if drug release is matrix diffusion controlled. Zero order is difficult to achieve with this device because the polymer matrix is insoluble, so as some drug dissolves and diffuses out, the diffusional path length increases for drug molecules more deeply embedded in the matrix.

Higuchi has also developed a spherical device model for homogeneous matrix.[60]

$$1 - 3\left(\frac{a'}{a_o}\right)^2 2\left(\frac{a'}{a_o}\right)^3 = \frac{6DC_s}{Aa_o^2}T = BT \tag{18}$$

Where

a_o = the radius of the whole pellet
a'= The radius of that part still unextracted

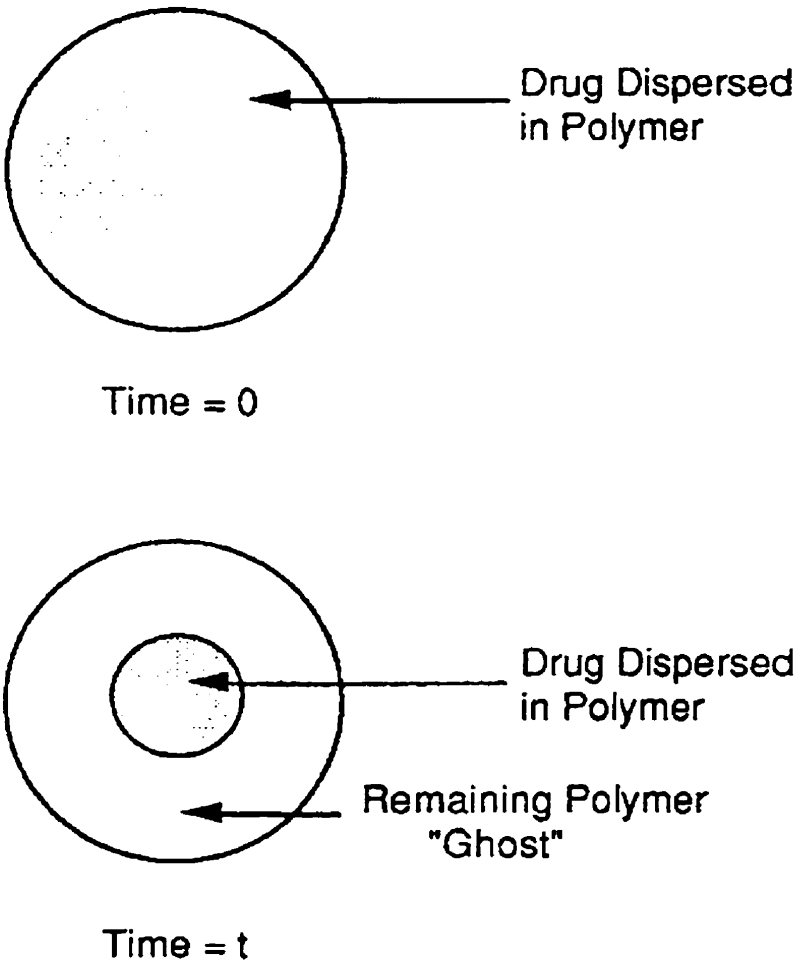


Figure 10: A matrix diffusional control delivery system, before release (time = 0) and after partial release (time = t). Ref (64)

1.3.5.3. DISSOLUTION AND DIFFUSION CONTROL

A combined dissolution and diffusion control of drug release can be accomplished by coating a drug core with a partially soluble membrane (Figure 11). Usually this membrane contains a combination of hydrophobic and hydrophilic polymers. The dissolution of the hydrophilic polymer causes the formation of pores through the membrane. This allows the dissolution media to enter the core and dissolve the drug. The fraction of the soluble polymer will be the dominant factor in controlling the release rate. The release rate is governed by the following equation:

$$\text{Release Rate} = AD\frac{(C_1 - C_2)}{\ell} \quad (19)$$

Where

A = surface area
D = diffusion coefficient of drug
C_1 = concentration of drug in the core
C_2 = concentration of drug in the dissolution medium
ℓ = diffusion path length

If the drug can diffuse through the membrane then Equations 12 to 14 as well as Equation 19 apply, depending on the systems geometry.

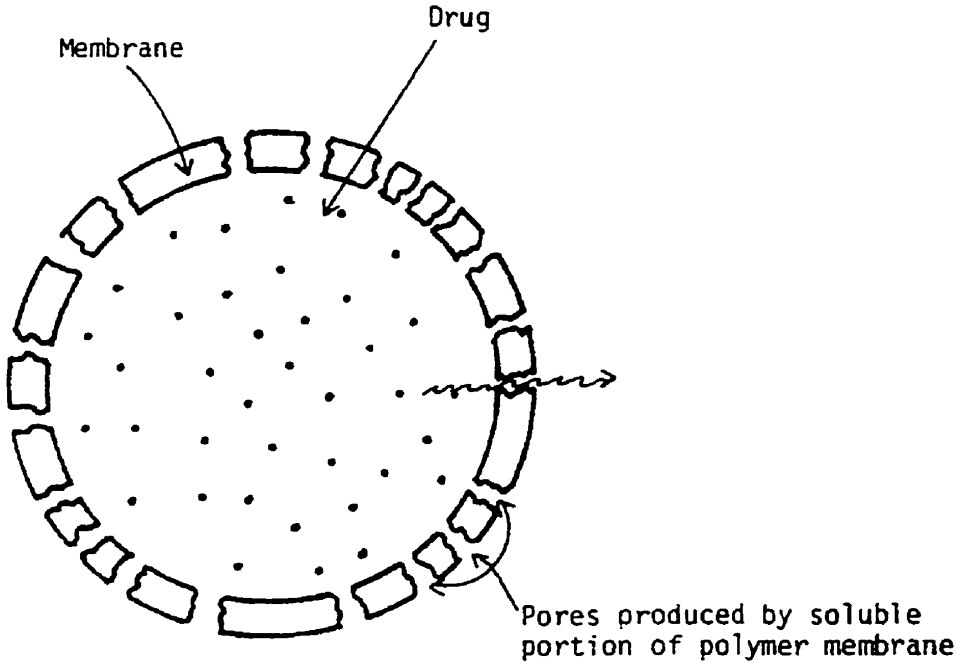


Figure 11: Schematic representation of diffusion and dissolution control of drug release. Ref (63)

1.3.5.4. ION EXCHANGE RESINS

Ion exchange resins are water insoluble cross-linked polymers containing salt forming groups in repeating positions on the resin chain. The salt forming functional group can contain acidic-reacting groups such as phenolic, carboxylic, or sulfonic called cationic exchange resins, or they can contain basic-reacting groups composed of either amino or quaternary ammonium groups called anionic exchange resins. The resins are formed by either incubating a drug solution with the resin or passing the drug solution through a column loaded with resin. After a suitable time period to allow complete displacement of the counter ion by the drug, the resin is washed and dried forming particles or beads. Drug release from the resin is facilitated by the exchange of the drug for a suitable counter ion from the dissolution medium of GI tract fluids as illustrated below:

Cationic ion exchange:

$$resin^- - drug^+ + Y^+ \rightarrow resin^- - Y^+ + drug^+$$

Anionic ion exchange:

$$resin^+ - drug^- + X^- \rightarrow resin^+ - X^- + drug^-$$

The release rate depends on the ionic environment of the GI tract as well as the resin properties. Since the electrolyte concentration is relatively constant, good reproducible release rates can be obtained. The extended releasing nature of the resins is the result of slow diffusion of the drug molecules through the resin particle structure after release by the counter ion. The area of diffusion, diffusional path length, and the rigidity of the resin govern the rate of drug diffusion. Therefore the degree of cross-linking, porosity, chemical composition, and the physical size of the particles can all be varied to modify

the release rate. An additional rate controlling measure is to microencapsulate the resin particles with a hydrophobic rate limiting polymer.[61, 62]

1.3.5.5. OSMOTICALLY CONTROLLED DEVICES

Recent advances in sustained and controlled drug delivery whether for oral administration or implantation, has focused on the use of osmotic pumps. These devices use osmotic pressure to deliver drug at a constant rate (Figure 12). The basic construction of an osmotic pump is a semipermeable membrane surrounding a core of osmotically active drug or a non-osmotically active drug with an osmotic agent (Figure 12B). The membrane allows free diffusion of water into the core but prevents the drug or the osmotic agent from diffusing out. A delivery orifice is drilled through the membrane by a laser or high speed mechanical drill to permit the dissolved drug's release. The system can also be designed without an orifice where the hydraulic pressure builds up until the device bursts releasing the entire contents (Figure 12C). Another type of pump is designed with an orifice and the drug in solution inside an impermeable membrane with the osmotic agent surrounding the bag (Figure 12A). The entire system is then further covered with a semipermeable membrane which allows the water to diffuse in, producing the hydraulic pressure which increase the pressure on the bag, forcing the drug solution out through the orifice. These devices, excluding the bursting type, deliver the drug at a zero order rate until the osmotically active ingredient drops below saturation solubility, after which the release follows a non-zero order rate.

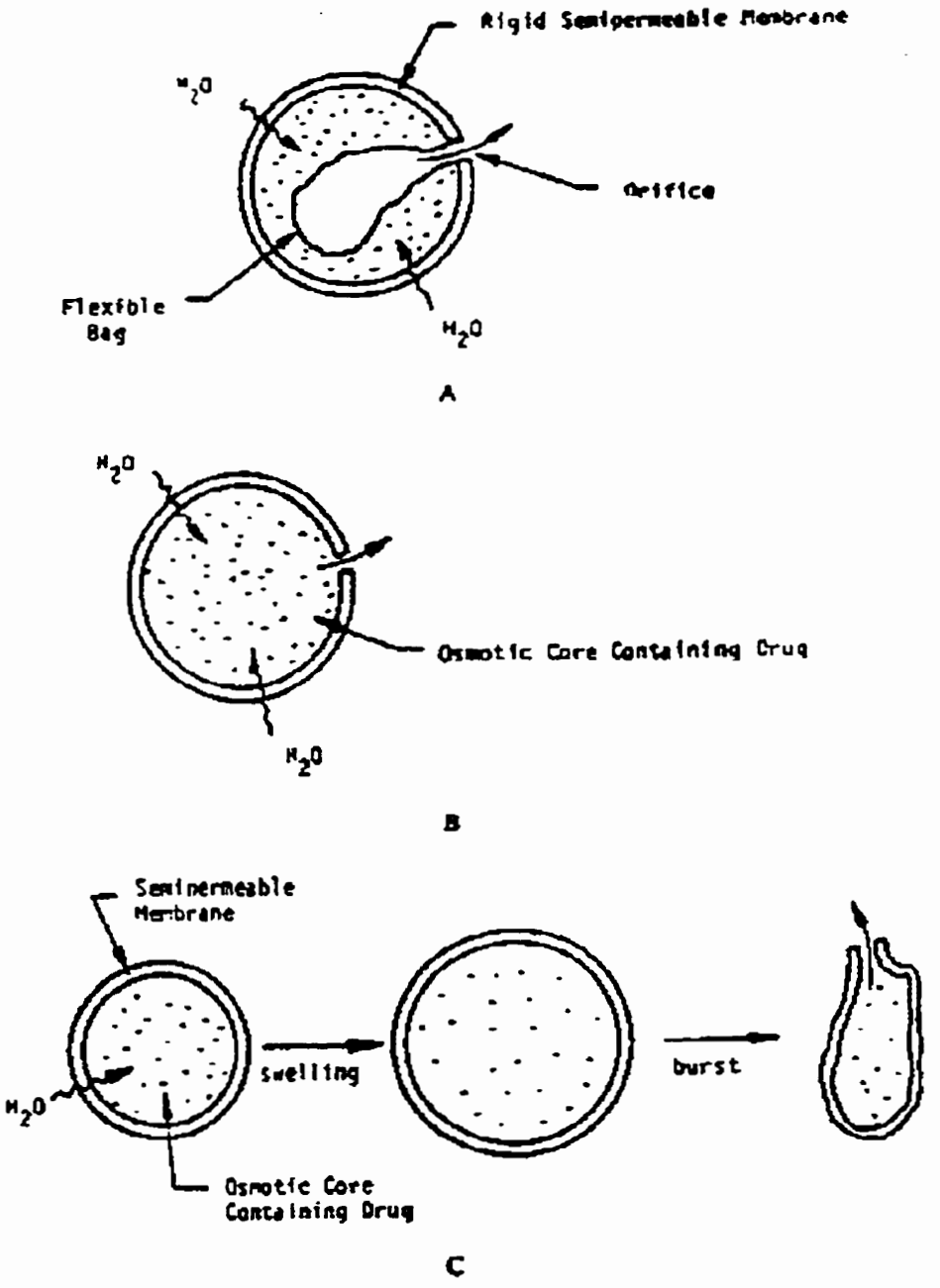


Figure 12: (A) Flexible bag osmotic pump. (B) Osmotic core containing drug (C) Osmotic pump containing no orifice. Ref (65)

1.4. MICROENCAPSULATION

Microencapsulation of drugs has other advantages in addition to eliciting a modified rate of release. Drugs like potassium chloride and Aspirin have been encapsulated with pH-dependent coatings to reduce gastric irritation.[49, 50] Flow properties of drugs can be enhanced by the coating, and the taste or odor of drugs can be masked.[50-68] Other advantages include increasing the ease of swallowing tablets and protecting labile materials from their environments. The microcapsules formed from this technique range in size from 1 to 200 μm.[48] Particles of less than 1 μm are termed nanoparticles, as they are measured in nanometers.[48] Depending on the operating conditions used during these processes, microcapsules can be produced in a variety of conformations and structures, with the mononuclear spherical shape being the most common (Figure 13).[48] The main processes employed to produce microcapsules are:

1. Simple or Complex coacervation / Phase separation
2. Interfacial polymerization
3. Solvent evaporation
4. Hydrophilic and hydrophobic congealing

Other methods of microencapsulation include air suspension coating and pan coating which involves the spraying of a polymer solution onto particles as small as granules or as large as tablets.[73]

1.4.1. THE CORE

The core material is usually comprised of one or more drugs with or without additives. Solid cores may consist of the drug as an agglomerate, or as the original powder or crystal. Liquid cores can be formed from the active component alone or from

the drug dissolved or dispersed in a solvent.[51,52] Additives include stabilizers. diluents. excipients, release rate retardants or accelerators. Poly(L-lactic acid) microspheres containing griseofulvin:phospholipid solid dispersions showed enhanced dissolution when compared to micronized griseofulvin.[53] Small cores tend to have aggregation problems during production due to the effect of surface attractive forces. and larger particles can have rapid sedimentation rates, while irregular shapes

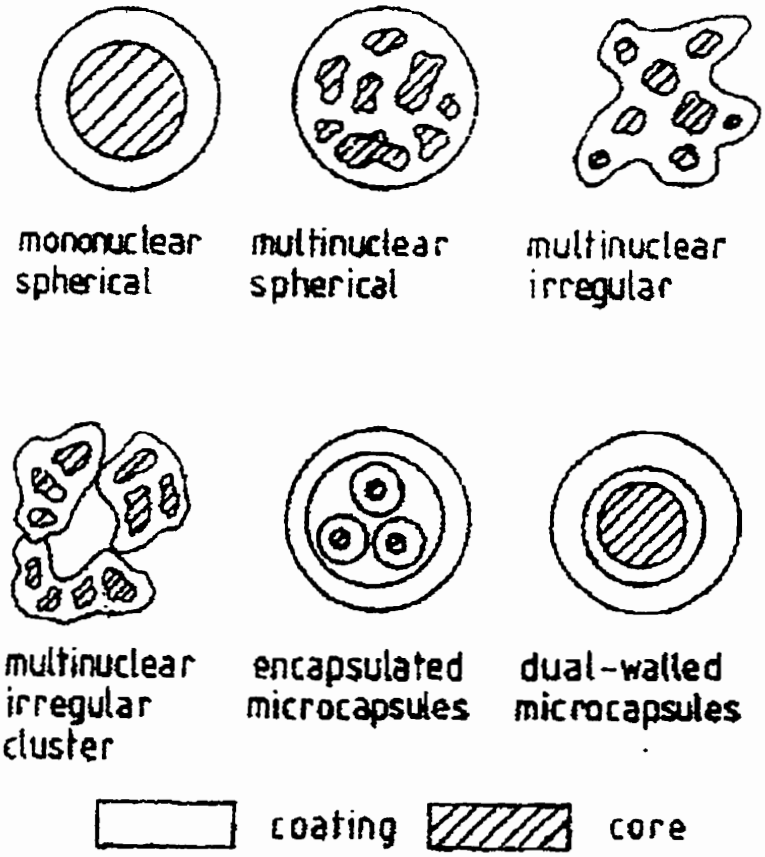


Figure 13: Some typical structures of microcapsules. Ref. (48)

can cause uneven distribution of the coating material.[48] Techniques such as size reduction, crystal habit modification, and agglomeration or spheronization have been employed to form particles of uniform size and shape that can be reproducibly microencapsulated. Another method is to use nonpareil seeds, which are usually sucrose pellets of uniform size and shape, to form the primary cores. These pellets are coated with a binder solution, usually PVP, then layered with the drug powder. These drug loaded pellets can then be microencapsulated utilizing one of the many techniques described previously.

1.4.2. THE COAT

There is a vast selection of coating materials that can be employed for microencapsulating drugs (Table 3). During production they can be used either alone or in combination with other polymers or additives. The choice of coat depends on the final use of the microspheres: enteric coating, biodegradable, inert or non-disintegrating, swelling, or porous. A good coating material should be capable of forming a film that binds with the core material and provides the desired coating properties, such as strength, flexibility, impermeability, and stability.[54] It should also be non-toxic, non-irritating, and non-allergenic, as well be compatible with the core material.[54]

1.4.3. SOLVENT

All of the techniques of microencapsulation listed in Table III, except melting-congealing, requires the use of an aqueous or non-aqueous solvent system to apply the coating material on the core (Table IV). The dissolution of the coating polymer by the

Table III: Some commonly used film formers for microencapsulation. Ref (48)

- Acacia
- Acrylic polymers and copolymers
  - polyacrylamide
  - polyacryldextran
  - polyalkyl cyanoacrylate
  - polymethyl methacrylate
- Agar and agarose
- Albumin
- Alginates
  - calcium alginate
  - sodium alginate
- Aluminum monostearate
- Carboxyvinyl polymer
- Cellulose derivatives
  - cellulose acetate
  - cellulose acetate butyrate
  - cellulose acetate phthalate
  - cellulose nitrate
  - ethylcellulose
  - hydroxypropylcellulose
  - hydroxypropylmethylcellulose
  - hydroxypropylmethylcellulose phthalate
  - methylcellulose
  - sodium carboxymethylcellulose
- Dextran
- Gelatin
- Poly (ε-caprolactone)
- Poly ester
- Polyethylene glycol
- Poly(ethylene-vinyl acetate)
- Polyglycolic acid, polylactic acid, and copolymers
- Polylysine
- Polystyrene
- Polyvinyl alcohol
- Polyvinylpyrrolidone
- Polyvinyl acetate phthalate
- Polyglutamic acid
- Shellac
- Starch
- Stearic acid
- Carnauba wax, Beeswax, Spermaceti, Paraffin wax

solvent is accomplished by the disruption of the cohesive forces between the polymer molecules. The more crystalline the polymer, the greater the cohesive forces, resulting in increased difficulty to dissolve the polymer. Increasing the solubility of the polymer results in an increase in the apparent viscosity of the system, from the uncoiling and extension of the polymer molecules. Decreasing the affinity of the solvent for the polymer causes aggregation and shrinkage of the polymer chain, reducing the apparent viscosity.

A high viscosity may cause problems with certain techniques of microencapsulation. In spray coating, plugging of the nozzle and poor spray patterns will yield non-uniform coats around the cores, as well as related mechanical problems. Poor solubility of the polymer inhibits the full extension of the lattice structure of the molecule thus decreasing good film forming properties. If the concentrations are quite low, increasing numbers of coating applications are required.

Solvent mixtures can improve dissolution properties as well as reduce the viscosity of the system in association with the concentration of the solid polymer.[56] The preferred solvent is one that has a low boiling point and heat of evaporation, so the process can be carried out at reduced temperatures and the solvent can be easily removed via evaporation.

Most solvents that have these properties are non-aqueous based solvents, such as aromatic, chlorinated hydrocarbons, or alcohols. The use of these solvents has led to serious environmental and heath concerns and hazards. Chlorinated hydrocarbons are potentially carcinogenic, while other organics are extremely flammable or explosive. Monitoring the final product for residual solvents is essential to ensure the product is not

adulterated. The cost of this process is usually higher due to the higher purchase price and the cost of disposal of these solvents. Aqueous polymeric coating has been gaining popularity over the organic method, as there is no risk of fire or explosion, the solvent is inexpensive, has lowest toxicity, and is non-polluting.

Table IV: Selected solvents used in microencapsulation, with physical data. Ref (56).

Solvent	Boiling point (°C) at 1013 mbar	Evaporation number[a]	Heat of evapo-ration (J/g)	Vapor pressure at 20°C (mbar)	MAK/ TLV ppm (mL/m³)	Minimum odor detection (mg/m³)	Ignition temper-ature (°C)	Flash point (°C)	Flammability range at 760 torr (vol %)
Ethanol	78.3	8.3	855	60	1000	93	425	+16	3.5–15.0
Methanol	64.7	6.3	1102	128	200	7800	508	+ 6.5	5.5–26.5
Isopropanol	82.3	11.0	667	40	400	50	634	+15	2.0–12.0
Acetone	56.2	2.0	520	240	1000	770	540	−19	2.5–13.0
Dichloromethane	40.2	2.0	321	475	100	550	605	No	13.0–22.0
Trichloromethane	61.2	2.5	247	210	10	1000	—	No	No
Water	100.0	60.0	2264	17.5	No	Very high	Very high	No	No

[a]Diethyl ether = 1.

Finely divided colloidal dispersions are classified into two categories based on the production techniques: True latex or Pseudolatex. True latex is produced by emulsion polymerization of a monomer or a monomer blend, which is then emulsified in an aqueous medium with the aid of an anionic or nonionic surfactant. Polymerization is then induced by an initiator that functions by free-radical, anionic, or cationic polymerization mechanisms. True latexes are limited to synthetic polymers of liquid insoluble monomers that can be emulsified in water.[56]

Pseudolatexes can be prepared from any existing thermoplastic water insoluble polymer, such as ethylcellulose or cellulose acetate phthalate. The polymer is dissolved in a suitable organic solvent and emulsified in water utilizing either sodium lauryl sulfate or cetyl alcohol as a stabilizer. After homogenization, the organic solvent is evaporated until the dispersion contains 30% solid material. Both true latexes and pseudolatexes are colloidal dispersions containing spherical solid or semisolid particles of less than 1 μm in diameter, yielding a milky, opaque fluid.

The advantages of these collodial dispersions are that water is the solvent and the varying lengths of the polymer chain has no effect on the extent of the apparent viscosity of the final product. Therefore the dispersion can be used at much higher concentrations than the corresponding solution using organic solvents. The disadvantages of aqueous coatings are the limited applicability due to the solubility, adhesivity, and stability either from the polymer or the core material. Also the process requires more time during production when the solvent is being removed because of the high heat of vaporization of water compared to organic solvents.

1.4.4. ADDITIVES

There are many additives that can be incorporated into the solvent film-former mixture to help improve the microcapsule's production, appearance, or plasticity. A plasticizer is added to a polymer to increase segmental mobility, impart flexibility, reduce brittleness, and to increase the resistance of the film coat to mechanical stress.[48] The plasticizer reduces the cohesive forces between the polymer molecules by interposing itself in the polymer chain. Good plasticizers tend to have structural features similar to

the polymer they are plasticizing, which helps in the chemical association of the two species.

An important prerequisite is that the plasticizer must be soluble in the same solvent system used for the film former. If not, separation of the two compounds may occur during evaporation of the solvent. Another property of plasticizers is in the ability to alter the permeability of a coating. Since the plasticizer is located in the interstices of the polymer, the addition of a hydrophilic plasticizer will increase the coat's permeability to water, and the converse will occur following the addition of a hydrophobic plasticizer.

Another additive is an "anti-tack" agent, used to reduce adhesion and friction of the core material during coating. "Anti-tack" agents are usually fatty acids, such as stearic acid.

Colorants enhance the appearance of the product, and help in identification.

1.5. MICROENCAPSULATION TECHNIQUES

1.5.1. COACERVATION/PHASE SEPARATION

Coacervation or phase separation was one of the first techniques developed for microencapsulation of pharmaceuticals. It involves the induction of a colloidal dispersion to form colloid-rich and colloid-poor regions by controlling temperature, pH, or electrolytes. The general process is outlined in Figure 14. There are two main types of coacervation, simple or complex.

Simple coacervation involves the use of one colloid usually gelatin dispersed in water. During coacervation the removal of polymer associated water occurs by

incorporation of agents with a higher affinity for water such as various alcohols and salts. The dehydrated colloid then aggregates with surrounding polymeric molecules to form the coacervate. The droplets collect on the surface of the drug cores, followed by fusion of these droplets to form a continuous layer. Hardening of the coat is accomplished by the addition of formaldehyde or other hardening agents.

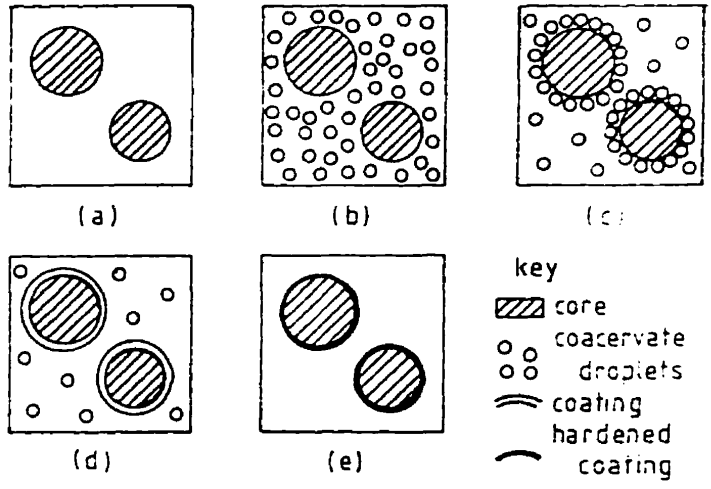


Figure 14: Typical steps in a coacervation method of microencapsulation. (A) Core particles dispersed in solution of polymer by agitation. (B) Coacervation visible as droplets of colloid-rich phase induced by one or more agents. (C) Deposition of coacervate droplets on surface of core particles. (D) Mergence of coacervate droplets to form the coating. (E) Shrinkage and crosslinking of the coating to rigidize it as necessary. Ref (48)

Gelatin coacervation is the most commonly used aqueous based process utilizing simple coacervation. Other materials include albumins, pectins, alginates, casein, agar, carboxymethylcellulose, and starchs. The limitation of the process is that water soluble cores cannot be used since the core would dissolve in the solvent during the coacervation

process. Non-aqueous systems have been developed to allow water soluble cores to be encapsulated. The polymers used for non-aqueous coacervation must dissolve in that vehicle. The disadvantage of these systems is that non-aqueous solvents can potentially reside in the particles after production, leaving trace residuals in the final product.

Complex coacervation involves the use of more than one colloid forming agent such as a gelatin-acacia mixture. The coacervate is formed from the interaction between two opposite charged polyelectrolytes. The interactions are usually induced by altering the pH of the medium to bring about ionization of the two polymer species. This charge neutralization results in the loss of bound water that in turn reduces the solubility and dispersability of the colloids. The coacervates formed then undergo the same process as shown in Figure 14. Many other polyelectrolyte combinations can be used such as albumin-alginate, alginate-gelatin, and pectin-gelatin.[69, 70]

Coacervation whether simple or complex can also be used to encapsulate liquids as well as solids. A novel approach is the use of acacia-gelatin coacervates to microencapsulate liposomes to help protect their degradation by bile salts and facilitate their oral delivery.[71]

1.5.2. INTERFACIAL POLYMERIZATION

Interfacial polymerization or polycondensation involves the polymerization of a monomer or several monomers at the interface of two immiscible substances. A water-in-oil or oil-in-water emulsion is made using a suitable surfactant. The dispersed phase contains a monomer with the core material dispersed or dissolved. The continuous phase contains a monomer or a crosslinking agent, which is usually added after the initial

emulsion is formed. The particle size is controlled by the degree of emulsification of the dispersed phase. The degree of polymerization can be controlled by using different monomers and crosslinking agents.

The application of interfacial polymerization for commercial products has not received much attention mainly because of the potential toxicity from any unreacted monomers and polymerization byproducts. Another problem with this system is that the limitations on polymer selection are based on those compounds that can polymerize under the right conditions to produce a pharmaceutical product.

1.5.3. SOLVENT EMULSIFICATION/EVAPORATION

The main difference between solvent evaporation and the first two microencapsulation techniques described previously is that the microspheres produced by solvent evaporation contain primarily the drug dispersed throughout a matrix. The other two techniques produce microspheres that are encapsulated by an additional coat. The former two techniques can be used to encapsulate liquids as well, whereas solvent evaporation methods cannot.

The solvent evaporation process involves the preparation of a solution of polymer and drug either dispersed or dissolved into a suitable low boiling solvent. This solution is then emulsified into a solvent that has a higher boiling point then the original dispersing solvent containing the drug and polymer. Emulsifiers are usually added into the continuous phase to aid in the emulsification process, then the system is subjected to heat and/or vacuum to remove the original dispersing solvent. This results in a suspension of microspheres that can then be removed by filtration or centrifugation.

The main disadvantage of this system is the use of organic solvents in the dispersion phase. Their low boiling points make them ideal solvents for this technique.[72] Another technique also called solvent evaporation involves dissolving the polymer and drug in a suitable solvent and evaporating the solvent. This results in a polymer mass throughout which the drug is intimately dispersed. The mass is then reduced to the desired particle size by comminution.

1.5.4. HYDROPHILIC AND HYDROPHOBIC CONGEALING

The congealing process involves the conversion of a polymer from the sol to the gel state. This change can result from the cooling of a hot dispersion of the polymer, or chemical solidification. Hydrophilic or hydrophobic congealing defines the physical properties of the polymer.

Hydrophobic congealing requires various waxes, fats, and oils (Table II). The drug is either dissolved or dispersed throughout the melted wax. The molten mixture is then dripped or poured into molds, which are rapidly cooled to solidify the wax. A major requirement for this process is that the drug must be stable at the temperatures needed to melt the wax. The microspheres produced are primarily of the matrix type that can be bioerodible or inert depending on the material used for their production.

Hydrophilic congealing utilizes polymers, which are soluble in water such as gelatin, agar, agarose, starch, alginates, chitosan, and polyethylene glycol. The drug is dispersed into the colloid which is then dropped into a coolant, causing the colloid to congeal. The coolants are usually liquids cooled below the colloid's sol-gel transition temperature and should not dissolve the drug or the colloid.

Typical coolants are petroleum ether, ethyl acetate, and mineral oil. Alginates and chitosan are sols that undergo spontaneous ionotropic gelation instead of requiring sudden temperature changes to induce gelation. Chitosan is a polysaccharide derived from chitin, which is found in many shellfish and various insects. It undergoes gelation when exposed to multivalent anions such as tripolyphosphate.[74] It can also be gelled when exposed to low pH.[75,76]

Alginate is extracted from giant brown seaweed such as *Macrocysitis pyrifera* and also from horsetail kelp like *Laminaria digitata.* A very important property of the alginates is that they are known to be nontoxic when taken orally, since alginates are used widely in the food and pharmaceutical industry as thickeners and stabilizers. Alginate is a linear polysaccharide comprised of alternating 1, 4 linked residues of β-D-mannuronic (M) and α-L-guluronic acid (G). These residues form two kinds of homopolymeric blocks - MM and GG - and one heteropolymeric block - MG.[77] These blocks are then organized in random order into a chain where their proportions vary with source and state of maturation of the plant (Figure 15). Sodium alginate is the water soluble sodium salt of alginic acid. Sodium alginate can undergo gelation in the presence of divalent metal ions, such as barium, strontium, calcium, or magnesium.

The main site for ion binding in alginates has been determined to be the GG-blocks. The conformation of the GG-blocks seems to allow easy access for ions to interact compared to the other blocks (Figure 15).[77] The MM and MG blocks do interact with the ions, but not as strongly as the GG blocks.[77] When the alginate strands interact with a divalent ion, such as calcium, strontium, or barium, significant inter and intra-

chain binding occurs. This has lead to the "egg box" model of co-operative binding between alginate strands (Figure 16).[79]

The production of alginate gels for pharmaceutical purposes has been mainly concentrated on calcium induced gelation, probably because of the potential toxicity with strontium or barium ions. The addition of calcium ions to a solution of sodium alginate results in an ion exchange, with the calcium ions displacing the sodium ions.[77] The ion exchange is governed by the equilibrium: (Alg = uronic acid residues)

$$2NaAlg + Ca^{2+} \Leftrightarrow CaAlg_2 + 2Na^{+}$$

This results in the formation of calcium alginate beads. Calcium alginate beads undergo pH sensitive reswelling in more basic medium and thus can help protect acid labile drugs from the low pH environment of the stomach.[80] At low pH values the alginate gel is insoluble because of the nonionized carboxylic acid groups which prevent solubilization.[81] As the pH increases to a more alkaline level, such as that in the intestines, alginate undergoes a base catalyzed β-elimination reaction.[82,83] This hydrolysis results in water entry to facilitate swelling of the xerogels and eventually the erosion and disintegration of the bead.

The production of calcium alginate beads begins with the preparation of a sodium alginate solution. This is the step for incorporation of any drug or substances needing entrapment in the gel. Next the gelling solution is prepared, usually a solution of calcium chloride, although calcium acetate or any source of calcium ions may be used. The alginate solution is then placed in a syringe, pipette, or any device, which is capable of administering the alginate to the gelling solution in a drop wise manner (Figure 17).

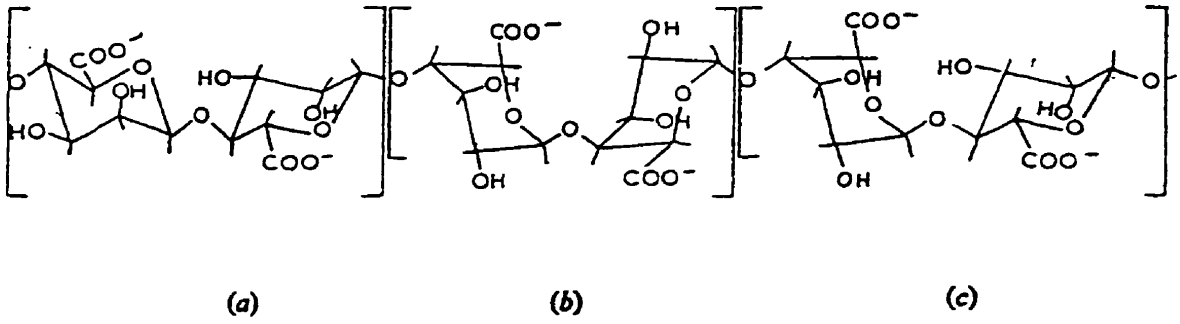


Figure 15: Schematic formula of alginate. (A) polymannuronate (B) polyguluronate (C) poly(mannuronosylguluronate) sequences. Ref (78)

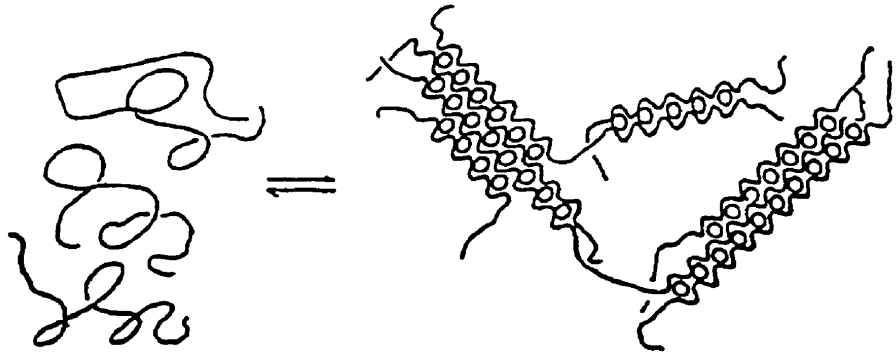


Figure 16: Schematic representation of "egg box" co-operative binding. Ref (78)

Due to the highly viscous nature of the alginate solution, some external force is required to push the alginate solution through these devices, as the force of gravity is usually not sufficient. Forces used include air pressure, or plunger type syringes.

Once the alginate is flowing in a drop wise manner, the gelling solution is gently stirred. In the calcium bath, the alginate sphere instantly exchanges Na^{+} for the Ca^{2+} ions. A white opaque border surrounding each transparent core can be observed as this occurred. As the sphere is allowed to reside in the bath, diffusion of calcium ions into the core of the bead eventually turns it completely white and opaque. This is a result of the ion exchange gradient set up by calcium ions penetrating the alginate matrix. The beads are allowed to cure in the gelling bath, then removed by filtration and rinsed to remove any unreacted calcium. They are allowed to dry and eventually form hard, irregularly or spherically shaped beads.

Calcium alginate beads were selected for study in this project because they are relatively easy to produce, composed of non-toxic material, and have an aqueous based production. Furthermore, their release properties exhibit an inherent enteric release without further coating, and facilitate a prolonged release of encapsulated drugs.

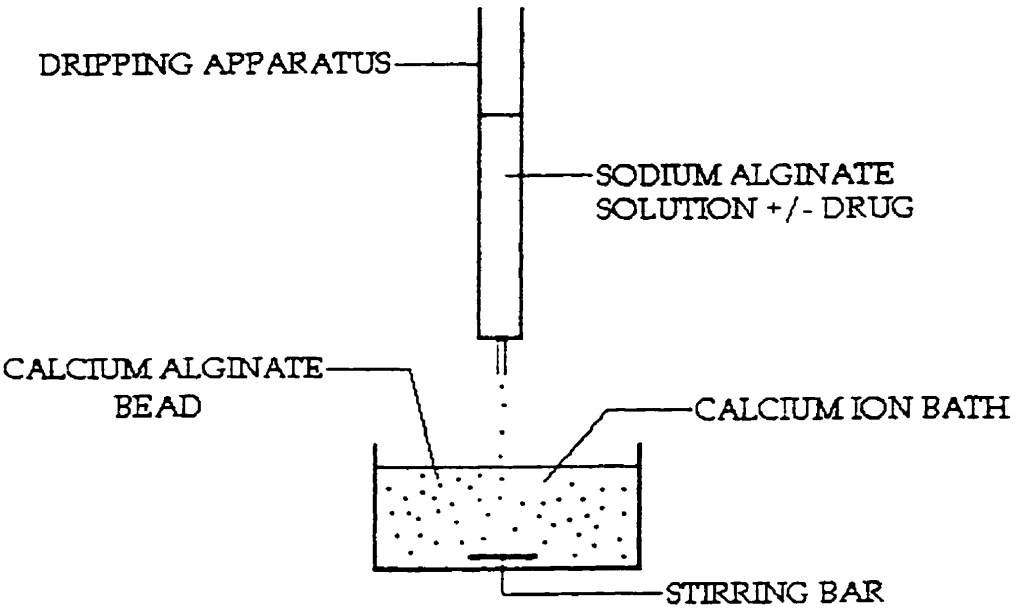


Figure 17: Schematic representation of the apparatus used to prepare calcium alginate beads.

1.6. HYPOTHESIS

The orally active iron chelator, DMHP, will be incorporated into calcium alginate beads to help facilitate a prolonged release.

The objectives of this study are as follows:

1. Assess a method for producing calcium alginate beads.

2. Evaluate production parameter changes, such as viscosity grade and concentration of sodium alginate, addition of additives, curing time, and drying temperature.

3. Characterize the morphology, size, shape, as well as the release patterns of calcium alginate beads in acidic and basic dissolution medium.

CHAPTER II

EXPERIMENTAL

2.1. MATERIALS AND EQUIPMENT

2.1.1 MATERIALS

1. Sodium alginate low, medium, and high viscosity grade, Sigma Chemical Co. (St. Louis, MO, USA)
2. Citrus pectin, N.F. Pharmaceutical grade. S. B. Pennick and Co. (New York)
3. 3-Hydroxy-2-methyl-4-pyrone (Maltol), Sigma Chemical Co. (St. Louis, MO, USA)
4. Methylamine (40% aqueous solution), Mallinckrodt Inc. (Paris, Kentucky, USA)
5. Calcium Chloride dihydrate, Mallinckrodt Inc. (Paris, Kentucky, USA)
6. Potassium Chloride, Mallinckrodt Inc. (Paris, Kentucky, USA)
7. Hydrochloric acid, Baxter Corporation (Toronto, Ont. Canada)
8. Sodium Hydroxide, Mallinckrodt Inc. (Paris, Kentucky, USA)
9. Ethanol 95%, Fisher Scientific Co. (Fair Lawn, New Jersey, USA)
10. Potassium phosphate (monobasic), Mallinckrodt Inc. (Paris, Kentucky, USA)
11. Sodium phosphate (dibasic, anhydrous), Mallinckrodt Inc. (Paris, Kentucky, USA)
12. Propylene Glycol, Mallinckrodt Inc. (Paris, Kentucky, USA)

13. Activated Charcoal (acid washed), Sigma Chemical Co. (St. Louis, MO, USA)

14. Phosphoric acid, Mallinckrodt Inc. (Paris, Kentucky, USA)

15. Potassium hydroxide, Mallinckrodt Inc. (Paris, Kentucky, USA)

16. Hydroxypropylmethylcellulose (HPMC), E10M. A gift from the Dow Chemical Co. (Midland, Michigan, USA)

17. Empty hard Gelatin capsules sizes 00, 0, 1, 2, and 3 T.U.B. Enterprises, (North Augusta, ONT.)

2.1.2. EQUIPMENT

1. Corning hot plate magnetic stirrer, Corning Glass Works (Corning, New York, USA)

2. Fisher Accumet pH meter, model 600. Fisher Scientific Company (Fair Lawn, New Jersey, USA)

3. Six unit Dissolution testing apparatus with gold plated USP dissolution test baskets. Vander kamp 600, Van-Kel Industries, (New Jersey, USA)

4. UV-Spectrophotometer, Shimadzu model UV-160, Shimadzu Corporation, (Kyoto, Japan)

5. USP standard sieves, Endecotts (test sieves) Limited. (London, England)

6. Oven, Precision Scientific Co. model 70 and 4. (Chicago, IL, USA)

7. Balance, Mettler AE 160. (Zurich, Switzerland)

8. Rotavapor, Buchi Laboratoriums-Technik AG, Switzerland

9. Propeller Type stirrer, Precision Scientific Co., (Chicago, IL, USA)

10. Centrifuge, IEC HN-SII, International Equipment Co. (Needham, MA, USA)

2.2. METHODOLOGY

2.2.1. SYNTHESIS OF 1,2-DIMETHYL-3-HYDROXYPYRID-4-ONE

The synthesis of 1,2-dimethyl-3-hydroxypyrid-4-one (DMHP) was adapted from the method published by Kontoghiorghes and Sheppard (Figure 18).[84] In a large round bottom flask, 75 g of maltol (3-hydroxy-2-methyl-4-pyrone) was dissolved in 1.5 L of deionized water. To this, 56 g of aqueous methylamine (40%) was added along with several boiling chips. The mixture was refluxed for 6.5 h not including heat up time.

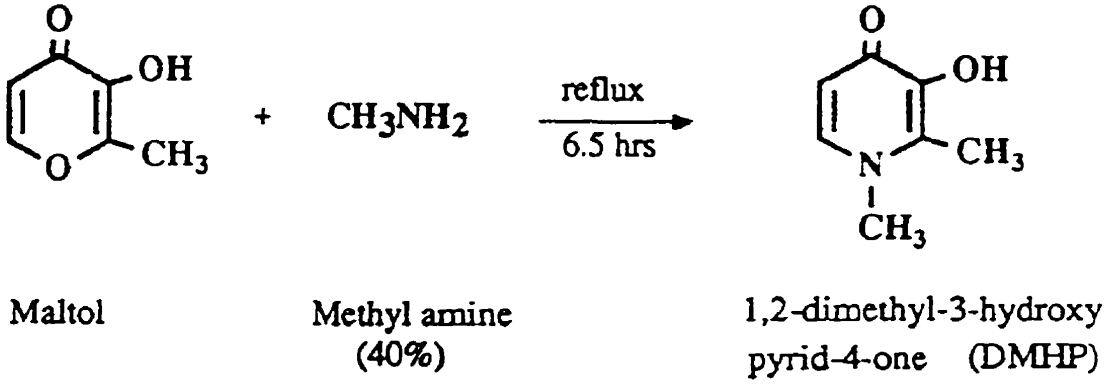


Figure 18: Schematic representation of DMHP synthesis.

The resulting brown solution was allowed to cool to 40-50^{0}C, then 3-5 g of activated charcoal was added and it was allowed to stand for 30 min. The slurry was then filtered using a buchner funnel assembly to remove the charcoal. The volume of the dark brown filtrate was reduced to ~200 mL by evaporating at 70^{0}C under vacuum using a rotary evaporator. The brown solid that precipitated out from the concentrate was filtered and saved in a separate beaker. The remaining filtrate was reduced even further using the

rotary evaporator followed by the precipitate being filtered off. This process was continued until all the filtrate was removed. The brown solid was collected and repeatedly crystallized in hot deionized water until white needle shaped crystals were obtained. The crystals were then dried to constant weight in a hot air oven at 110^0C. The purity of DMHP was monitored by determining the melting point for each batch. A random batch was subjected to NMR analysis to confirm the structure and purity of the DMHP.

2.2.2. PREPARATION OF DISSOLUTION MEDIA

2.2.2.1. KCl-HCl BUFFER SOLUTION (pH = 2.0)

To 1.3 L of a 0.2 M HCl solution was added 74.55 g of KCl. When the KCl was dissolved the solution was made up to 20 L with deionized water. The pH was checked and adjusted to pH = 2.0 as required with either concentrated HCl or concentrated KOH solutions.

2.2.2.2. PHOSPHATE BUFFER SOLUTION (pH = 7.4)

The two salts, 35.73 g of monopotassium phosphate and 152.07 g of disodium phosphate anhydrous were weighed and dissolved in ~15 L of deionized water. The resulting solution was then made up to 20 L and the pH checked and adjusted to 7.4 as required with either concentrated phosphoric acid or a concentrated solution of NaOH.

2.2.3. SPECTROPHOTOMETRIC ANALYSIS OF DMHP

A stock solution of DMHP (10 g/L) was prepared in each buffer. Serial dilutions were done to achieve a concentration range of 1 - 20 μg/mL. Using a 1 mL: 1 cm path length quartz spectrophotometer cell, the absorbance of DMHP was determined at a λ_{max} = 278 nm in phosphate buffer pH = 7.4 and λ_{max} = 274 nm in HCl-KCl buffer pH = 2.0. The data was plotted and fitted using linear regression. The linear regression equation was determined for DMHP in phosphate buffer pH = 7.4 and HCl-KCl buffer pH = 2.0 for the purpose of quantifying unknown concentrations of DMHP.

2.2.4. PREPARATION OF CALCIUM ALGINATE BEADS

In a 250 mL beaker sufficient DMHP was dissolved in 200 mL of deionized water to yield a final concentration of 15 mg/mL. Sodium alginate was added to the DMHP solution to yield a concentration of 1%, 2%, 2.5% w/v medium viscosity grade (~3500 cps), or 3%, 3.5% w/v low viscosity grade (~250 cps). Table V displays the various formulations prepared. The dispersion of the alginate was achieved using a propeller type stirrer whose efficiency was not affected by the range of viscosities of the various alginate grades and concentrations.

A 1 L solution of 15 mg/mL DMHP was also prepared, 500 mL was placed in a separate beaker for a rinsing solution and the other 500 mL was used as the curing solution. To the curing solution, $CaCl_2$ was added to give a concentration of 4 %w/v. The solution was put into a large flat bottom glass container containing a magnetic stir bar. The solution was gently agitated by placing the container on a magnetic stirrer.

The alginate/DMHP solution was added into the curing solution with the aid of a peristaltic pump flowing through size 16 tygon tubing and dripping out a 1 mm I.d. glass dropper. The dropper was positioned ~30 cm above the curing solution and the drops were positioned to fall away from the swirling center. Acceptable dripping was achieved when there was a uniform spherical bead falling from the glass dropper. The alginate/DMHP solution dripped until all was added, this took on average 8 min.

Gentle agitation was continued for an additional 15, 30, 45, or 60 min. to allow beads to cure further. After curing, the beads were removed from the bath by filtering through a Buchner funnel apparatus. The beads were then removed and placed in a beaker to which 250 mL of the 15 mg/mL rinsing solution of DMHP was added. The beads were agitated using a glass stirring rod to facilitate rinsing of the beads to remove unreacted calcium ions. This was done for 1 min. at which time the beads were filtered again and the rinsing process repeated one more time.

Surface moisture was removed by placing the beads between two sheets of paper towel and gently patting dry. The beads were then split into two equal portions by weight. One portion was tray dried at 40^0C and the other tray dried at 60^0C. Both batches were dried for 24 h. The beads were harvested separately and sieved through USP standard sieves to remove surface DMHP and characterize the size distribution. This method was used except for modification by the incorporation of various additives. A list of the various formulations produced is given in Table VI.

Pectin 1, 2, or 3% w/v was added to 200 mL of a 15 mg/mL DMHP solution and dispersed. The pH was then adjusted to 10 using concentrated NaOH to ionize the pectin. Low viscosity grade sodium alginate was then added to give a final concentration of 3%

w/v. The same procedure was used previously for bead preparation except that only the 60 min curing time was used.

As another additive, 20 mL of propylene glycol was added to a 200 mL DMHP 15 mg/mL solution followed by low viscosity grade sodium alginate to give a concentration of 3% w/v. The beads were prepared as described previously, but only allowed to cure for 30 min.

A slightly modified method was used when HPMC E10M was incorporated into the beads. The required amount of DMHP was weighed out to give a 15 mg/mL solution in 200 mL of deionized water. One hundred mL of boiling water was used to dissolve the DMHP first, then HPMC E10M was added to give a concentration of 0.5% w/v in 200 mL. The mixture was stirred using a propeller type stirrer until the HPMC had completely dispersed. Then 100 ml of deionized water chilled to 4^0C was added to facilitate the dissolution of the HPMC. The mixture was allowed to stir for 20 min. before sodium alginate (low viscosity grade) was added to give 2 or 3% w/v concentrations. Once all the ingredients were completely dispersed, the solution was allowed to cool to room temperature and to permit the air bubbles to dissipate. The beads then were prepared as before except curing was done for 30 or 60 min. time periods.

Table V: Composition of formulations prepared (N=3).

Formulation No.	Composition	Curing Time (min.)	Drying Temp (°C)
1a4	1% w/v alginate (med)	15	40
1a6	1% w/v alginate (med)	15	60
1b4	1% w/v alginate (med)	30	40
1b6	1% w/v alginate (med)	30	60
1c4	1% w/v alginate (med)	45	40
1c6	1% w/v alginate (med)	45	60
2a4	2% w/v alginate (med)	15	40
2a6	2% w/v alginate (med)	15	60
2b4	2% w/v alginate (med)	30	40
2b6	2% w/v alginate (med)	30	60
2c4	2% w/v alginate (med)	45	40
2c6	2% w/v alginate (med)	45	60
3a4	2.5% w/v alginate (med)	15	40
3a6	2.5% w/v alginate (med)	15	60
3b4	2.5% w/v alginate (med)	30	40
3b6	2.5% w/v alginate (med)	30	60
3c4	2.5% w/v alginate (med)	45	40
3c6	2.5% w/v alginate (med)	45	60
4a4	3% w/v alginate (low)	15	40
4a6	3% w/v alginate (low)	15	60
4b4	3% w/v alginate (low)	30	40
4b6	3% w/v alginate (low)	30	60
4c4	3% w/v alginate (low)	45	40
4c6	3% w/v alginate (low)	45	60
5a4	3.5% w/v alginate (low)	15	40
5a6	3.5% w/v alginate (low)	15	60
5b4	3.5% w/v alginate (low)	30	40
5b6	3.5% w/v alginate (low)	30	60
5c4	3.5% w/v alginate (low)	45	40
5c6	3.5% w/v alginate (low)	45	60

Legend: Numbers 1 to 5 refer to the sequential increase in alginate concentration. Curing times of 15, 30, and 45 min. are designated as a, b, and c respectively. The drying temp is 4 for 40°C and 6 for 60°C.

Table VI: Composition of calcium alginate beads with the addition of additives (N=3).

Formulation No.	Composition	Curing Time (min.)	Drying Temp (°C)
6a	3% w/v alginate (low) + 1% w/v pectin	60	40
6b	3% w/v alginate (low) + 1% w/v pectin	60	60
7a	3% w/v alginate (low) + 2% w/v pectin	60	40
7b	3% w/v alginate (low) + 2% w/v pectin	60	60
8a	3% w/v alginate (low) + 3% w/v pectin	60	40
8b	3% w/v alginate (low) + 3% w/v pectin	60	60
9a	2% w/v alginate (low) + 0.5% E10M HPMC	30	40
9b	2% w/v alginate (low) + 0.5% E10M HPMC	30	60
9c	2% w/v alginate (low) + 0.5% E10M HPMC	60	40
9d	2% w/v alginate (low) + 0.5% E10M HPMC	60	60
10a	3% w/v alginate (low) + 0.5% E10M HPMC	30	40
10b	3% w/v alginate (low) + 0.5% E10M HPMC	30	60
11a	3% w/v alginate (low) + 20 mL propylene glycol	30	40
11b	3% w/v alginate (low) + 20 mL propylene glycol	30	60

2.2.5. PRODUCTION YIELD

The yield of calcium alginate bead production was determined using the following equation:

$$\%\text{Yield} = \frac{\text{Total weight of beads after drying at 40 or 60 }^{\circ}\text{C}}{\left(\frac{\text{Total weight of ingredients}}{2}\right)} x100$$

The total weight of the ingredients is the sum of the alginate, DMHP, and/or any additives. Since the beads were split into two equal portions by weight, it was assumed that half of the ingredients would be incorporated into each portion. The weight of the dried beads after sieving was determined for the two drying temperatures.

2.2.6. WEIGHT FRACTION AFTER CURING

Twenty drops of the alginate-DMHP solution were collected in mid stream of dripping and weighed. This weight is designated w_o or the original weight of the beads before curing. The rest of the solution was allowed to drip into the curing solution and remain there for the designated time. The beads were removed from the curing solution and rinsed according to the process described previously. Twenty hydrated beads were removed before the drying process in the ovens, and weighed. This weight is designated w_t, or the weight of the beads at the set curing time of 15, 30, or 60 min. The weight fraction is determined by the following equation:

$$\text{Weight Fraction} = \frac{w_t}{w_o}$$

2.2.7. PERCENT WEIGHT LOSS ON DRYING

The weight loss of the calcium alginate hydrogels after drying was measured by comparing the total weight of the hydrated beads to the total weight after drying at the two different drying temperatures. Only formulations 1 through 5 cured for 45 min. were evaluated for the comparison. Equation is as follows:

$$1-\left(\frac{\text{Weight of dry beads}}{\text{Weight of hydrated beads}}\right)x100$$

2.2.8. RESIDUAL WATER CONTENT

Shortly after being tray dried at 40^0C or 60^0C, twenty dried beads from each batch containing DMHP were weighted separately. Gravimetric analysis was performed on these beads after they were dried at 115^0C to constant weight

2.2.9. ENCAPSULATION EFFICIENCY

A suitable quantity of calcium alginate beads (~350 mg) were dissolved in 200 ml of pH 7.4 phosphate buffer with agitation for 24 h. The resulting solution was centrifuged, and then an aliquot of the supernatant was diluted and the concentration of DMHP measured spectrophotometrically at 278 nm. This was done for batches of beads dried at 40^0C and at 60^0C for 24h. Since the beads were split into two equal portions by weight, it was assumed that half of the original amount of the DMHP would be with each portion. The following equation was used to determine the percent encapsulation:

$$\%EE=\frac{\text{Amt in beads}}{1.5g}x100$$

Encapsulation was also compared as a function of yield, termed percent drug loaded by comparing the amount of DMHP in the beads to the total weight of the beads dried at 40 or 60^{0}C for 24h.

2.2.10. DISSOLUTION STUDIES

Dissolution tests were conducted using the USP apparatus #1 (basket) in 900 ml of KCl-HCl pH 2.0 buffer and 900 mL of phosphate buffer pH 7.4. A bead weight equivalent to 60 mg of DMHP was arbitrarily chosen and placed in a suitable size gelatin capsule. Dissolution was tested over a 6 h period at a stirring rate of 50 rpm and a temperature of 37^{0}C ± 0.1^{0}C. A sample size of 0.5 ml was withdrawn from the cell at pre-determined intervals and analyzed spectrophotometrically at 278 nm for pH 7.4, and at 274 nm for pH 2.0 to measure DMHP concentration.

CHAPTER III

RESULTS AND DISCUSSION

3.1. QUALITY CONTROL AND SYNTHESIS OF DMHP

The yield from the DMHP synthesis process ranged from 40-55%. Analysis of periodic batches by ^{1}H-NMR (300 MHz, D_2O, HOD 4.63) confirmed the structure of DMHP (Figure 19); δ 2.35 (3 H. s, 2-CH_3). 3.70 (3 H. s. 1-CH_3). 6.36 and 7.48 (2 H, Abq *J* 7 H_z, H-5 and H-6).[84] The melting points of the various batches ranged from 267-270^{0}C. which is consistent with the literature value of 266-268^{0}C.[30]

3.2. CALIBRATION CURVES FOR DMHP

Calibration curves of DMHP over the range of 1-20 μg/mL obeyed Beers law in both phosphate and KCL-HCL buffers (Figure 20). Linearity was very reproducible from different batches of DMHP. Regression analysis provided the following equations that were used to determine unknown concentrations of DMHP:

Phosphate pH = 7.4	Y = 0.089X + (-0.008)	r^2 = 0.9999
KCL-HCL pH = 2.0	Y = 0.05X + 0.0024	r^2 = 0.9999

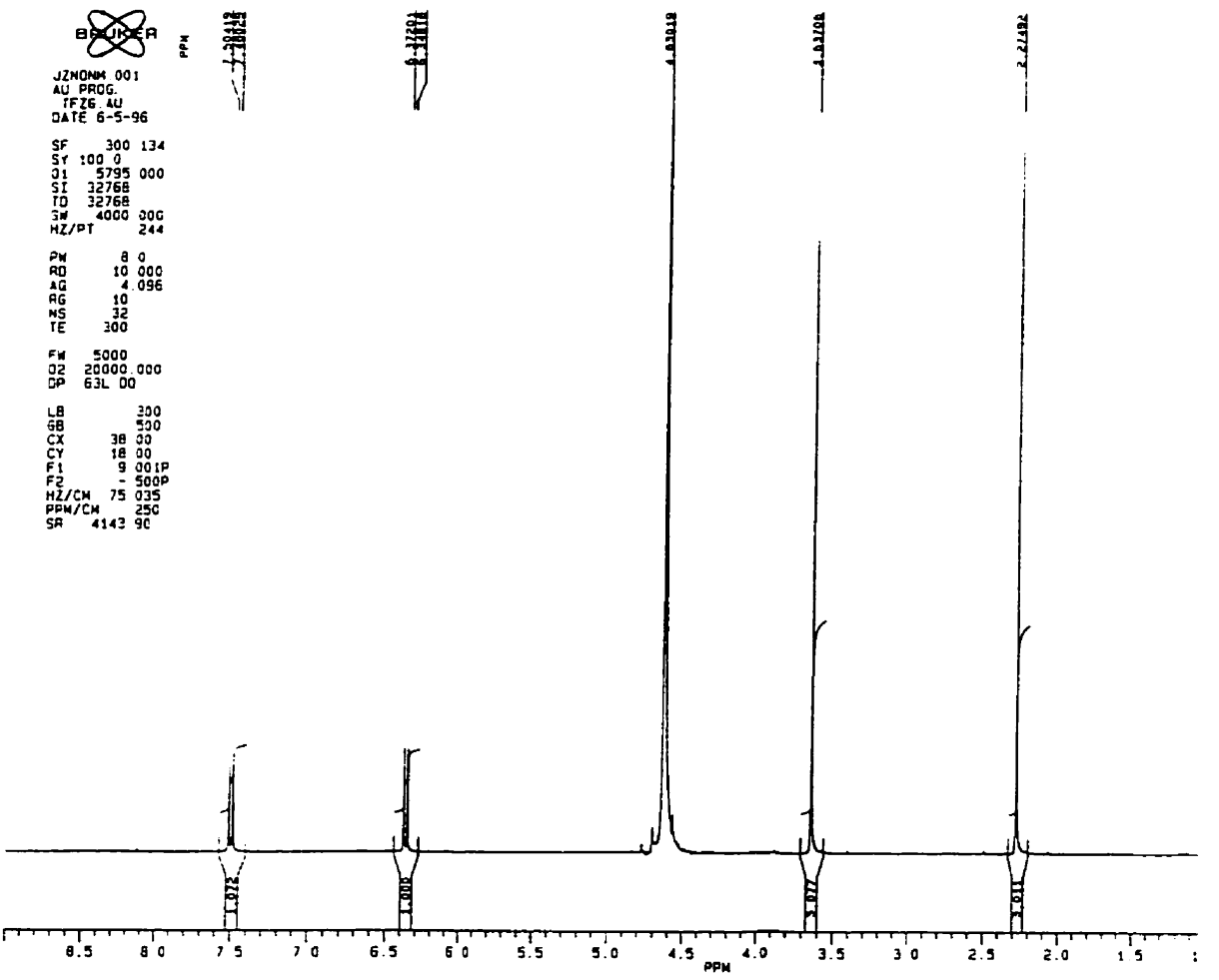


Figure 19: NMR of DMHP

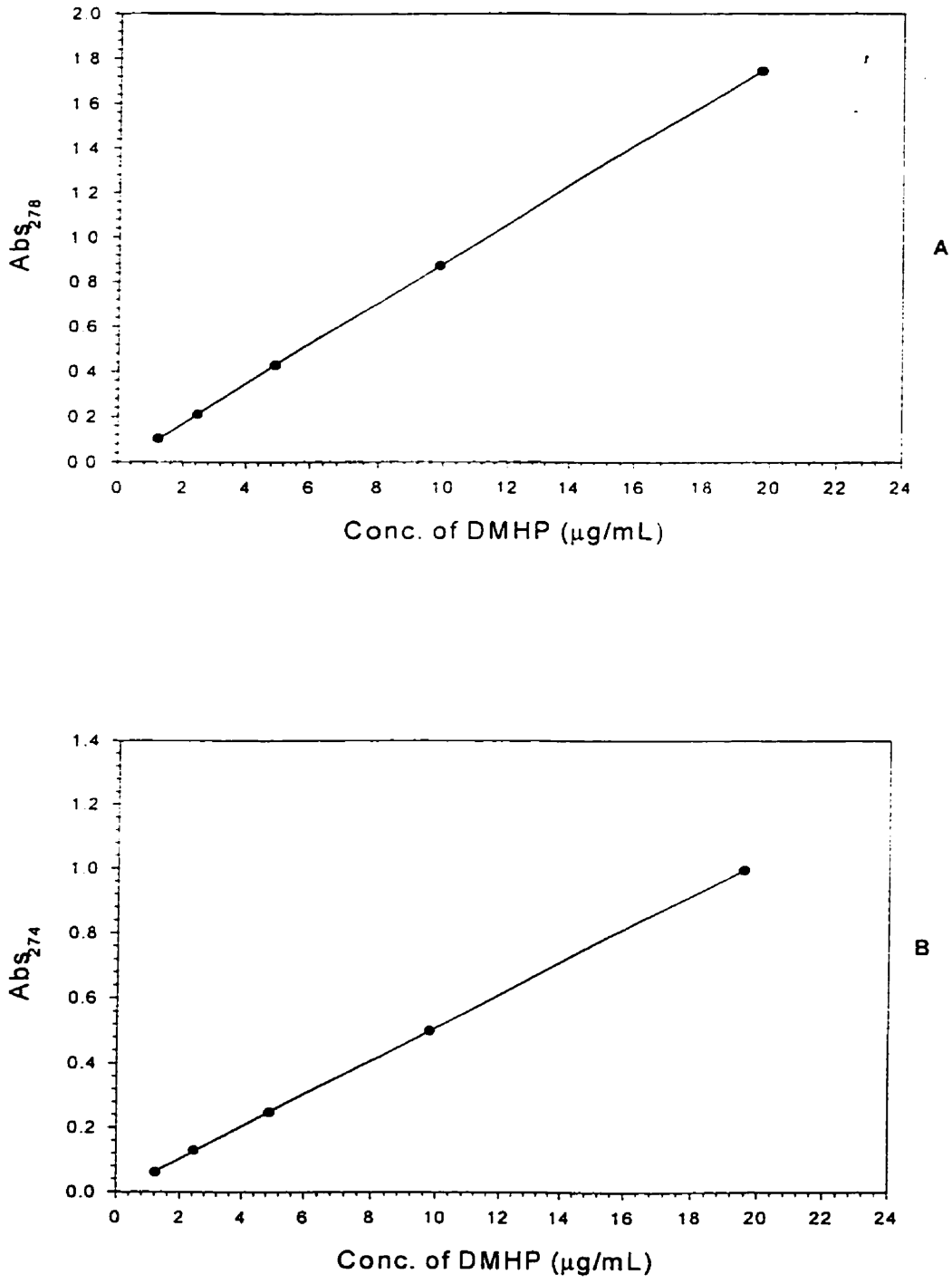


Figure 20: Beers plot of DMHP in A) phosphate pH=7.4 and B) KCL-HCL pH=2.0 buffer.

3.3. FORMATION OF CALCIUM ALGINATE BEADS

A very important factor to consider during the incorporation of DMHP into calcium alginate beads is whether or not it binds to the calcium ions during production. *In vitro* and *in vivo* work have demonstrated DMHP's specificity for iron and it's low affinity for calcium ions.[19,31,37] The assumption was made that any binding of calcium with DMHP will be negligible.

A concentration of 15 mg/mL DMHP was chosen, as higher concentrations exceeded the aqueous solubility and resulted in precipitation. To avoid any concentration gradient developing in the curing bath or in the rinsing solution, the same concentration of DMHP was added to both. This minimized any loss of DMHP out of the beads due to diffusion while the curing or rinsing process is taking place. Ostberg and Graffner found a 100-200% increase in encapsulation efficiency when the curing and rinsing solution were saturated with the drug being incorporated.[85] Patting the beads dry with paper towels helps to remove any surface DMHP solution. This prevents DMHP crystal formation on the surface of the bead during the drying process.

Using higher concentrations of sodium alginate or grades with higher viscosity caused plugging of the dropper tube or improper drop formation. Therefore only two viscosity grades, medium and low, at maximum concentrations of 2.5% w/v and 3.5 % w/v respectively could be used in these formulations. Incorporation of the various additives also produced some increase in the viscosity of the solution. A peristaltic pump apparatus was the most efficient method to deliver the alginate solution with or without additives at the various concentrations.

Upon contact with the calcium chloride solution, the sodium alginate drops instantly exchange Na^+ ions for Ca^{2+} ions. Visually one can see a white opaque border surrounding a transparent core where this occurs. Once the beads curing is complete the hydrated beads are round and opaque with no visual signs of DMHP crystals on the surface.

3.4. YIELD AND ENCAPSULATION EFFICIENCY

Table VII summarizes the yields of the production methods and the encapsulation efficiencies for the various formulations. The yield varied from 67 - 105% for beads dried at 40^{0}C and 76-101 % when dried at 60^{0}C. A 100 % yield would theoretically be expected to result in an encapsulation of 100%. The added weight may arise from residual water in the beads as the yield does not take into account the water content nor is it corrected for it. No discernable pattern can be seen in the yield when comparing the alginate concentration or drying temperatures.

The encapsulation efficiency (EE) based on theoretical input ranged from 12 - 75%. Beads dried at 40^{0}C usually had lower EE then beads dried at 60^{0}C. An exception was the beads prepared with pectin (formulations 6a,6b,7a,7b,8a,8b), which had similar EE's at the two drying temperatures. The appearance of DMHP crystals that were easily removed from the surface of beads dried at 40^{0}C following sieving may contribute to the low EE. At 60^{0}C, DMHP formed cakes on the bead surface that were not easily removed by sieving in formulations 1-5 (including all subunits) this may have contributed to the increase in EE and percent drug loaded. Furthermore, an increase in the alginate

concentration in formulations 1-5 produced an increase in the EE. The presence of more alginate strands seems to improve the entrapment of DMHP in the bead matrix.

Very high EE's were obtained with formulation 6a, 6b, 7a, 7b, 8a, and 8b and the beads did not have any surface caking of DMHP after drying. Here the increase in EE can be explained as a result of the tightening of the bead lattice when pectin undergoes ionotropic gelation along with the alginate. A slight increase of EE was also observed with increasing amounts of pectin. High EE's were also seen when HPMC was incorporated into the beads (formulations 9-10 including subunits) and dried at 60^0C. The lower EE's at 40^0C was due to crystals of DMHP forming on the surface of the beads after drying which were easily removed by sieving. No crystal clumps formed on the beads dried at 60^0C.

Another loss of DMHP in addition to that caused by drying at 40^0C is the effect of the curing step. While the beads cure they contract. This is brought about by the interaction of alginate with calcium. This contraction causes the expulsion of interior water and the DMHP that is dissolved in that water. Thus an encapsulation efficiency of 100% is difficult to achieve for this system when the drug is soluble in the aqueous medium.

3.5. SIZE DISTRIBUTION AND MORPHOLOGY

After drying, beads prepared with 1% sodium alginate medium viscosity achieved an irregular shape. The other sodium alginate concentrations with or without the various additives formed smooth-spherical beads. Bead color was a medium dark brown with white specks throughout representing the DMHP dispersed in the matrix. The fractions

of increasing bead size seems to increase proportionally with increasing amounts of alginate, with the majority being >12 mesh starting at 2.0 % sodium alginate (Figure 21, 22, 23). There does not seem to be any significant difference in the size of the beads when dried at the two different temperatures.

Beads dried at 40^0C always had a coating of DMHP crystals, which was easily removed after sieving. The beads dried at 60^0C formed a continuous coat of DMHP crystals and only some of it could be removed during sieving. However, most of this surface coat of DMHP on the beads could not be easily removed. The presence of DMHP crystals on the surface seemed to occur more with beads formed using a lower concentration of sodium alginate. Also, when additives such as pectin or HPMC were added, there was very little DMHP coating or individual crystals on the surface of the beads. When the beads were dried at 40^0C, the migration of the interior water to the surface brought DMHP to the surface causing the formation of crystals. When the beads were dried at 60^0C, the faster evaporation of the water after migration to the surface caused the formation of a solid coat of DMHP crystals.

Table VII: Yield and encapsulation efficiency of the various formulations.

Form. No:	(%)Yield		Amt of DMHP(mg)		EE (%)		% Drug loaded	
	40^0C	60^0C	40^0C	60^0C	40^0C	60^0C	40^0C	60^0C
1a	81.124	80.056	454.5	523.5	30.3	34.9	22.4	26.2
1b	70.812	79.603	219.0	498.0	14.6	33.2	12.4	25.0
1c	67.923	76.571	193.5	471.0	12.9	31.4	11.4	24.6
2a	93.333	93.442	487.5	658.5	32.5	43.9	14.9	20.1
2b	99.359	93.537	621.0	691.5	41.4	46.1	17.9	21.1
2c	87.494	93.712	387.0	688.5	25.8	45.9	12.6	21.0
3a	79.063	84.682	288.0	610.5	19.2	40.7	9.1	18.0
3b	82.878	89.154	336.0	663.0	22.4	44.2	10.1	18.6
3c	82.19	87.478	322.5	625.5	21.5	41.7	9.8	17.9
4a	90.907	91.761	550.5	738.0	36.7	49.2	13.5	17.9
4b	97.875	95.881	699.0	772.5	46.6	51.5	15.9	17.9
4c	94.232	92.853	639.0	721.5	42.6	48.1	15.1	17.3
5a	89.571	90.147	597.0	771.0	39.8	51.4	13.3	17.1
5b	89.887	92.683	514.5	768.0	34.3	51.2	11.4	16.6
5c	90.28	94.094	477.0	763.5	31.8	50.9	10.6	16.2
6a+b	104.65	99.649	952.5	949.5	63.5	63.3	16.5	17.3
7a+b	105.26	97.278	1042.5	1018.5	69.5	67.9	15.2	16.1
8a+b	105.73	96.689	1126.5	1078.5	75.1	71.9	14.2	14.9
9a+b	93.18	97.258	604.5	933.0	40.3	62.2	16.2	24.0
9c+d	88.47	95.208	400.5	861.0	26.7	57.4	11.3	22.6
10a+b	91.758	96.058	559.5	912.0	37.3	60.8	12.2	19.0
11a+b	99.798	101.36	577.5	826.5	38.5	55.1	11.6	16.3

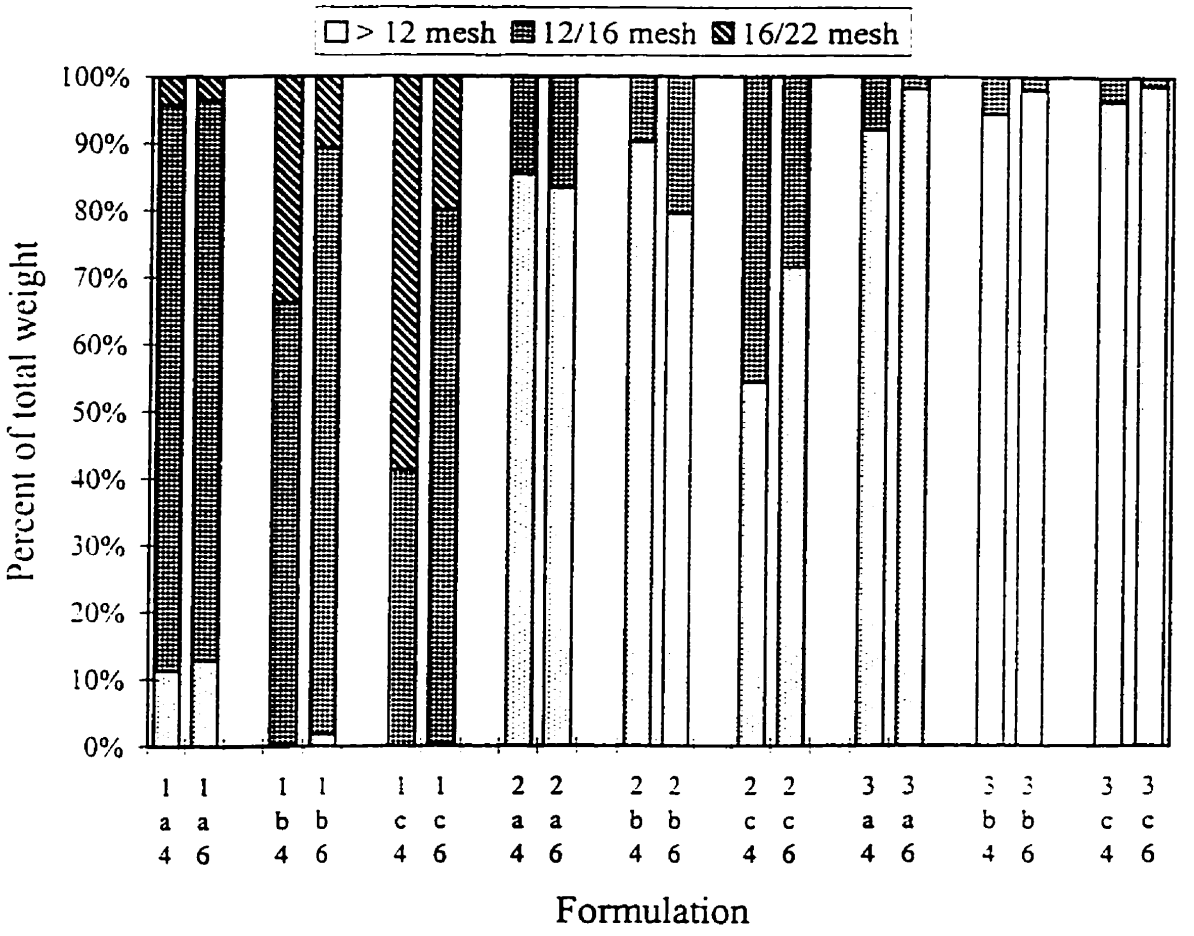


Figure 21: Size distribution of formulation 1-3. All prepared with 1%, 2% or 2.5% medium viscosity sodium alginate respectively. Curing time was 15, 30, or 45 min. (a, b, or c respectively). Drying temperature was either 40°C or 60°C (4 or 6).

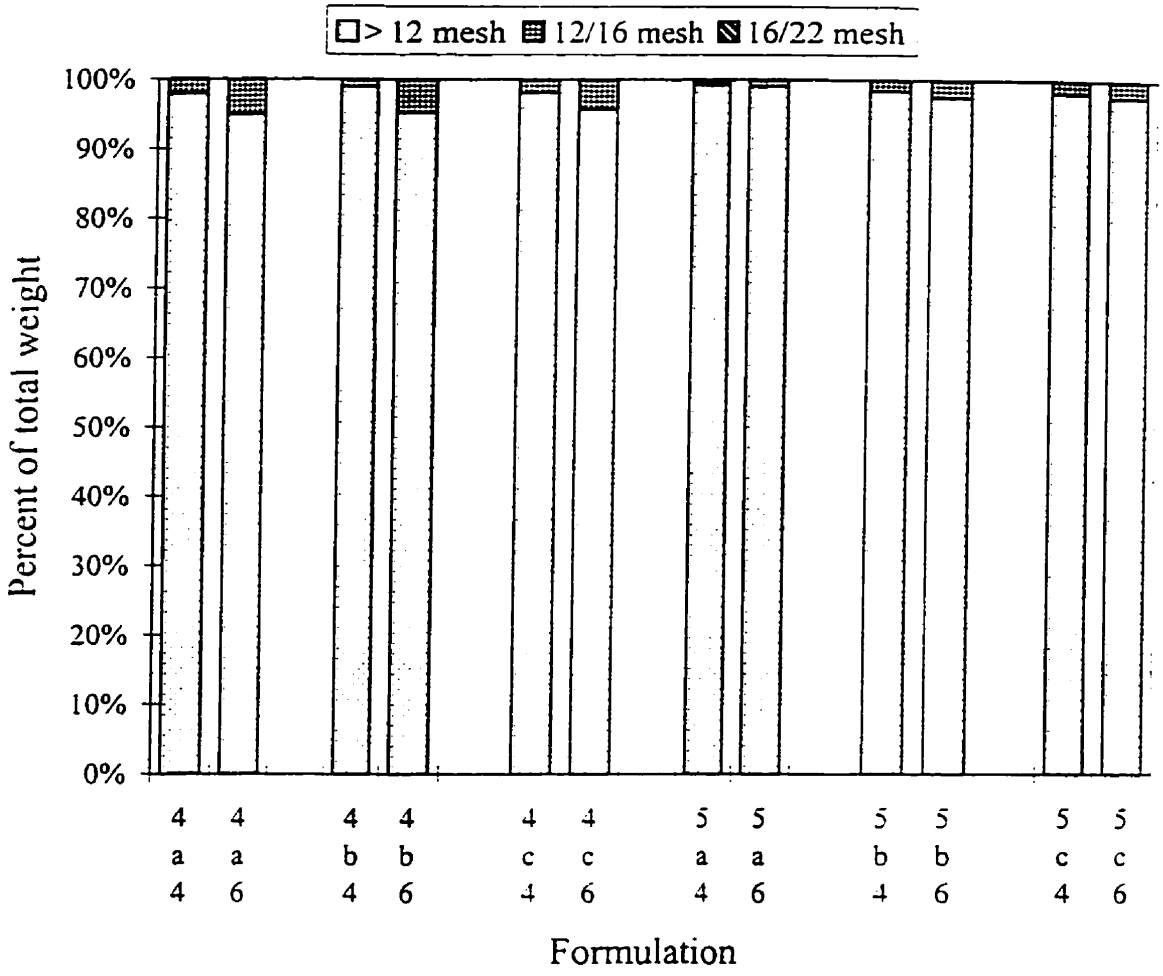


Figure 22: Size distribution of formulation 4 and 5. All prepared with 3% and 3.5% low viscosity sodium alginate respectively. Curing time was 15, 30, or 45 min. (a, b, or c respectively). Drying temperature was either 40°C or 60°C (4 or 6).

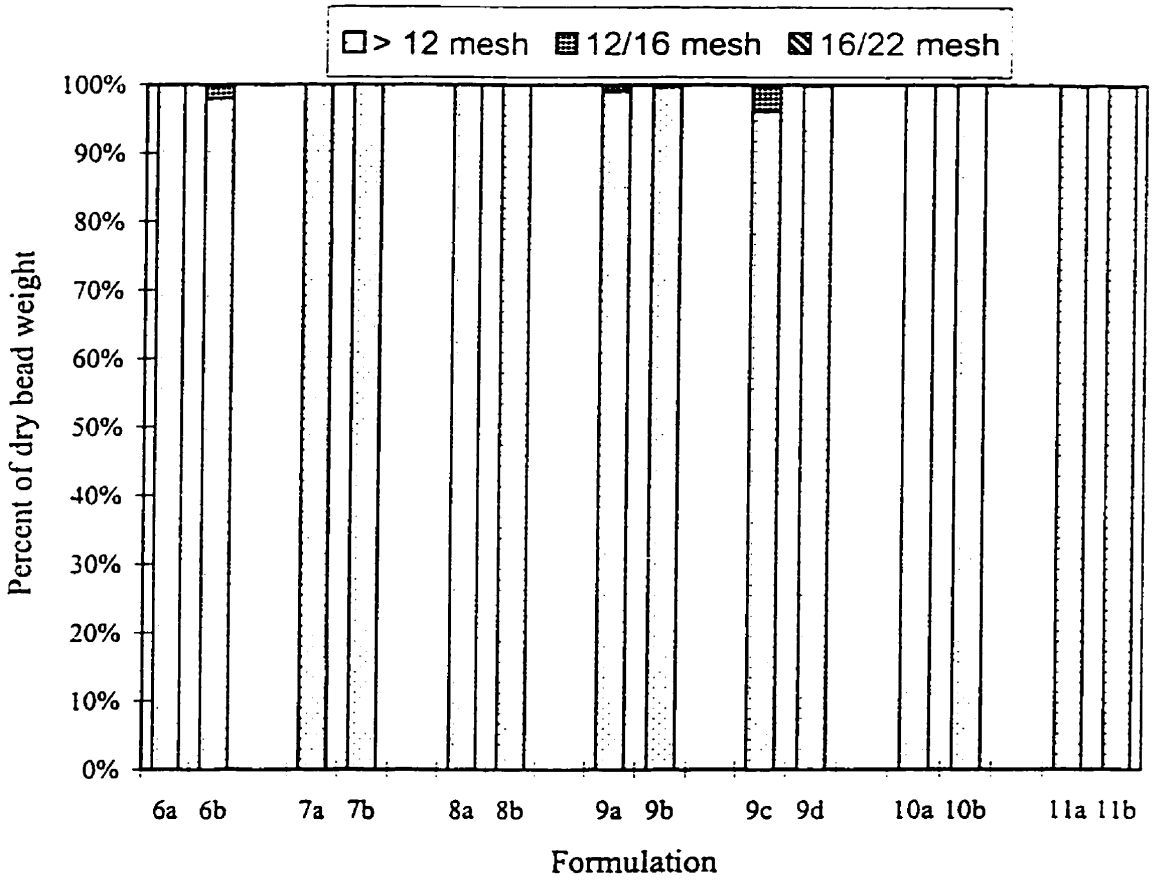


Figure 23: Size distribution of formulations 6-11. Formulations represent the addition of additives. See Table VI page 64 for legend on the various formulations.

3.6. WEIGHT FRACTION AFTER CURING

In the formation of microspheres, the process of alginate gelling involves the ion exchange of sodium ions for calcium ions. This occurs instantaneously when the alginate bead comes into contact with the calcium ion bath. A spherical bead is formed, and the rate of calcium reacting with the sodium alginate can be monitored by observing the white opaque surface surrounding the transparent core. This border slowly thickens as the calcium ions penetrate into the interior of the bead as the calcium reacts. As the gelling process occurs, water and solutes dissolved in it will be forced out of the interior. This was seen by Ostberg and Graffner who reported an increase in the calcium ion bath volume of between 15 - 50 mL after removal of the beads.[85] Therefore it is suggested that when bead curing is complete that the weight of the bead should be constant.[80] Figure 24 shows the weight fraction of formulations 1 through 5. A very small decrease in the weight fraction occurs throughout curing times ranging from 15 to 45 min. From these results it can be assumed that the beads achieve full curing by 45 min. Curing times of 60 min. were therefore used with subsequent formulations to provide a safety margin to ensure all beads were completely cured before removal.

3.7. PERCENT WEIGHT LOSS ON DRYING AND RESIDUAL WATER

In figure 25 is shown the graph of the weight loss on drying as a function of alginate concentration. The weight loss was inversely proportional to the alginate concentration. The beads dried at 40^0C should no difference in weight loss than when

dried at 60^{0}C. The results following 15 and 30 min. curing times yielded similar weight losses (data not shown). This suggests that beads achieved similar weights after drying at the two different temperatures. Surface DMHP crystals were easily removed from most formulations dried at 40^{0}C but DMHP cakes from 60^{0}C were not, so these cakes may contribute added to the bead weight, thus the percent loss may have been higher at 60^{0}C.

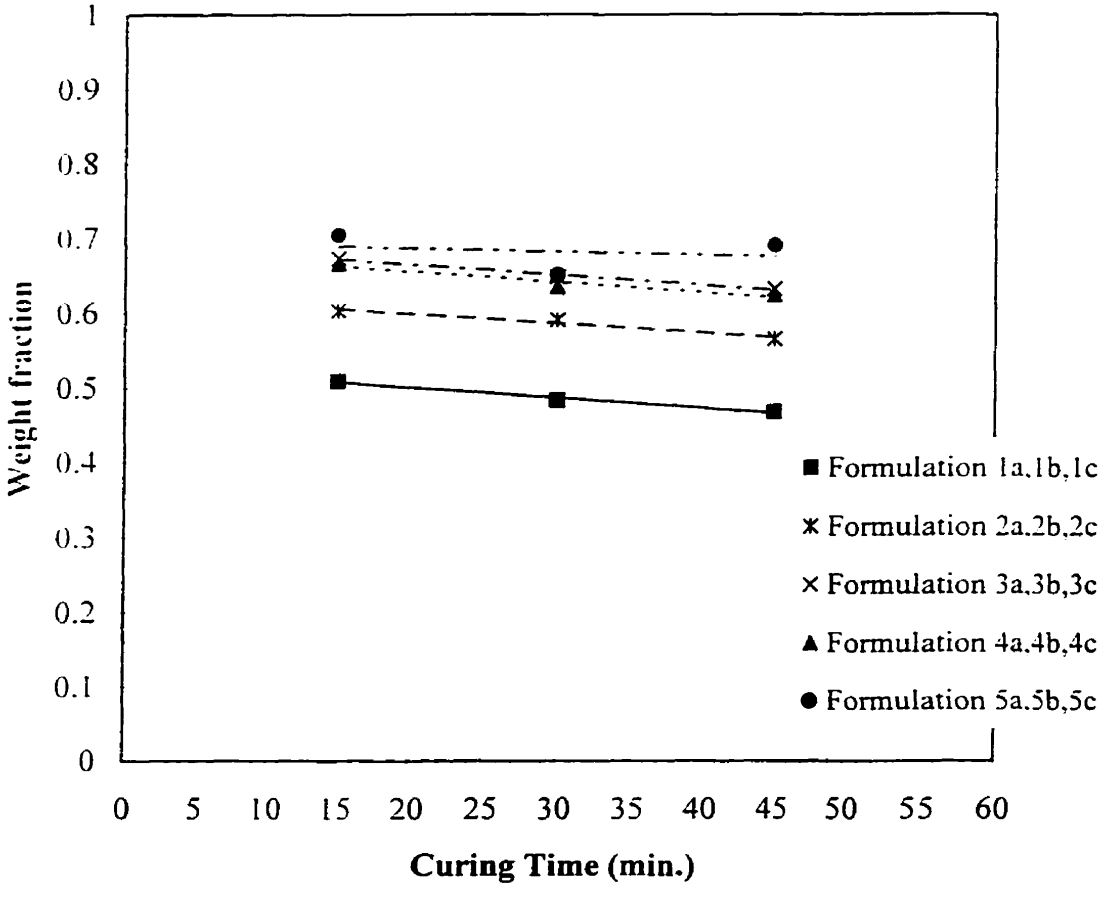


Figure 24: Weight fraction of beads after curing.

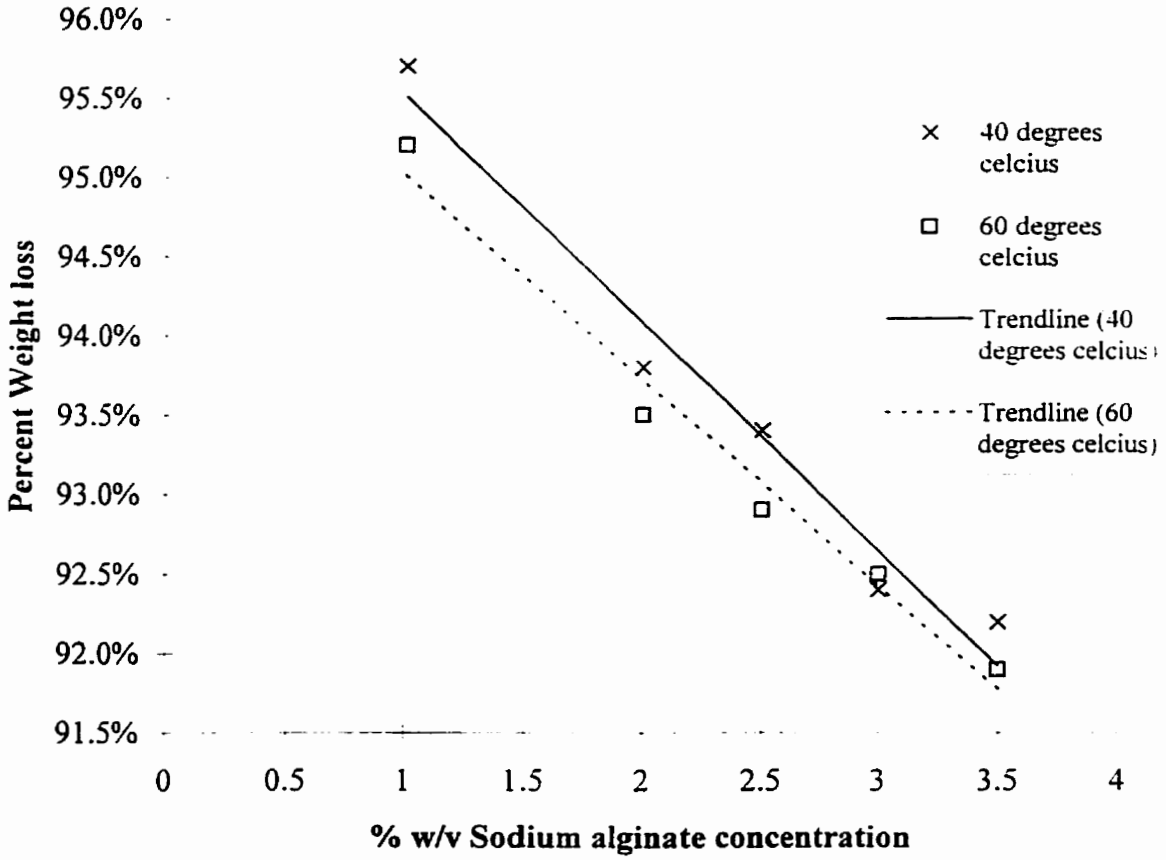


Figure 25: Percent weight loss after drying of alginate beads

The gravimetric analysis of dried beads provides information about the degree of water hydration after the beads have dried. Table VIII shows the results for the formulations after drying at 40^0C and 60^0C. Beads dried at 40^0C contained a higher percentage of water of hydration than beads dried at 60^0C, and the amount of hydration is independent of the alginate concentration.

Table VIII: Gravimetric analysis data

Formulation No.	Drying Temperature (^{0}C)	% loss on drying
1a4	40	10.7
1a6	60	6.2
1b4	40	10.1
1b6	60	5.9
1c4	40	10.0
1c6	60	5.9
2a4	40	11.9
2a6	60	6.4
2b4	40	10.8
2b6	60	5.7
2c4	40	9.2
2c6	60	5.4
3a4	40	9.2
3a6	60	6.7
3b4	40	10.7
3b6	60	8.1
3c4	40	9.2
3c6	60	6.9
4a4	40	11.2
4a6	60	6.8

Table VIII con't: Gravimetric analysis data

Formulation No.	Drying Temperature (^{0}C)	% loss on drying
4b4	40	11.1
4b6	60	6.6
4c4	40	10.9
4c6	60	6.8
5a4	40	9.7
5a6	60	6.3
5b4	40	9.8
5b6	60	6.6
5c4	40	10.2
5c6	60	6.9
6a	40	11.1
6b	60	8.0
7a	40	12.1
7b	60	8.8
8a	40	11.6
8b	60	7.7
9a	40	8.9
9b	60	4.3
9c	40	11.3
9d	60	5.3
10a	40	10.8
10b	60	7.6
11a	40	13.1
11b	60	10.1

3.8. *IN VITRO* DISSOLUTION TESTING OF ALGINATE BEADS

The U.S.P. guidelines for dissolution testing of modified release drug delivery systems requires the testing in a low pH medium for 2 h. followed by testing in a higher pH medium.[38] This procedure simulates the entry and residence of the delivery system in the stomach followed by emptying into the intestines. When the calcium alginate beads were tested using this protocol, release in pH = 2.0 buffer was virtually 100% within 20 min. Therefore the release profile of the beads could not be determined using the U.S.P. protocol. The release rate was therefore characterized individually in media at the low and high pH values.

The release of the DMHP from calcium alginate beads with no additives in pH=2.0 HCl-KCl buffer is shown in Figures 26-31. Visual observation of the beads after the six hours of dissolution testing revealed that the beads were not swollen and remained intact and were still very firm. There was no difference in the release rate with respect to alginate concentration, curing time, or drying temperature for the five formulations when compared to DMHP powder alone. There are two reports demonstrating cracks and fissures in the alginate bead structure.[85,86] These fissures and cracks are probably the cause of DMHP's fast release, as the dissolution medium is allowed to penetrate the bead and dissolve the DMHP. Since the DMHP molecule is very small and water soluble, the pore size does not exhibit any occlusive properties in preventing the diffusion of DMHP. Pfister et al observed a fast release pattern of highly water soluble compounds in dried calcium alginate matrices compared to low solubility compounds.[87] The release of indomethacin in low pH was negligible as the compound is not soluble at low pH, even though it is a relatively small molecule.[88] Furthermore, a large compound such as blue

dextran (mw = 2 000 000) which is moderately soluble in water. displayed a release of only 10-30% in pH=1.2 buffer as it is to large to diffuse through the pores and cracks of the dry alginate matrix.[89] The goals of a prolonged release delivery system is to provide minimal release in the stomach, and provide a sustained release as it transcends the intestines. Thus compounds with high solubility and/or low molecular weight can not be retained from releasing initially in the stomach even though the bead matrix does not swell or erode here.

In pH = 7.4 phosphate buffer, the beads appeared to swell shortly after contact with the medium. As the dissolution continued, the beads slowly eroded until there was no solid remaining in the basket after the six hours. In figures 32-37, the release patterns of DMHP encapsulated calcium alginate beads in phosphate buffer pH = 7.4 are shown.

Plots of percent release of DMHP versus time follow parabolic and sigmodial shape curves. Within 15-30 min., at all the curing times and drying temperatures, the beads released 30-55% of DMHP. Beads made with 1% medium viscosity alginate showed a continuous parabolic curve regardless of production parameters. The extent of release is lower when they are cured for 30 and 45 min. compared to 15 min., when dried at 40^0C. When dried at 60^0C the release is similar over the three curing times and is higher than beads cured for 30 and 45 min. dried at 40^0C. The beads prepared with 2% medium viscosity alginate is parabolic and similar in release to 1% alginate when cured for 15 min. for both drying temperatures. When cured for 30 and 45 min. they begin to display some sigmodial shape. This sigmodial shape is consistent for the remaining alginate concentrations and viscosity grades. The sigmodial shape is attributed to the initial water entry into the beads dissolving the DMHP causing the rapid initial release

within 15-30 min.. followed by a swelling of the outer surface of the bead as it hydrates. This hydration forms a barrier around the bead that reduces the release of DMHP and helps prolong the release. The bead also erodes while it continues to hydrate and the interior of the bead swells. Beads prepared with 3% alginate low viscosity showed the best release at curing times of 30 and 45 min. dried at 40^0C. Their release is lower then when dried at 60^0C at the same curing times. Although beads made with 3.5% low viscosity alginate showed better release rates when cured for 15 min. at the two drying temperatures than 3 % alginate, the 3% was better at the 30 and 45 min. curing times. The rate of release in general is more prolonged from beads dried at 40^0C than at 60^0C.

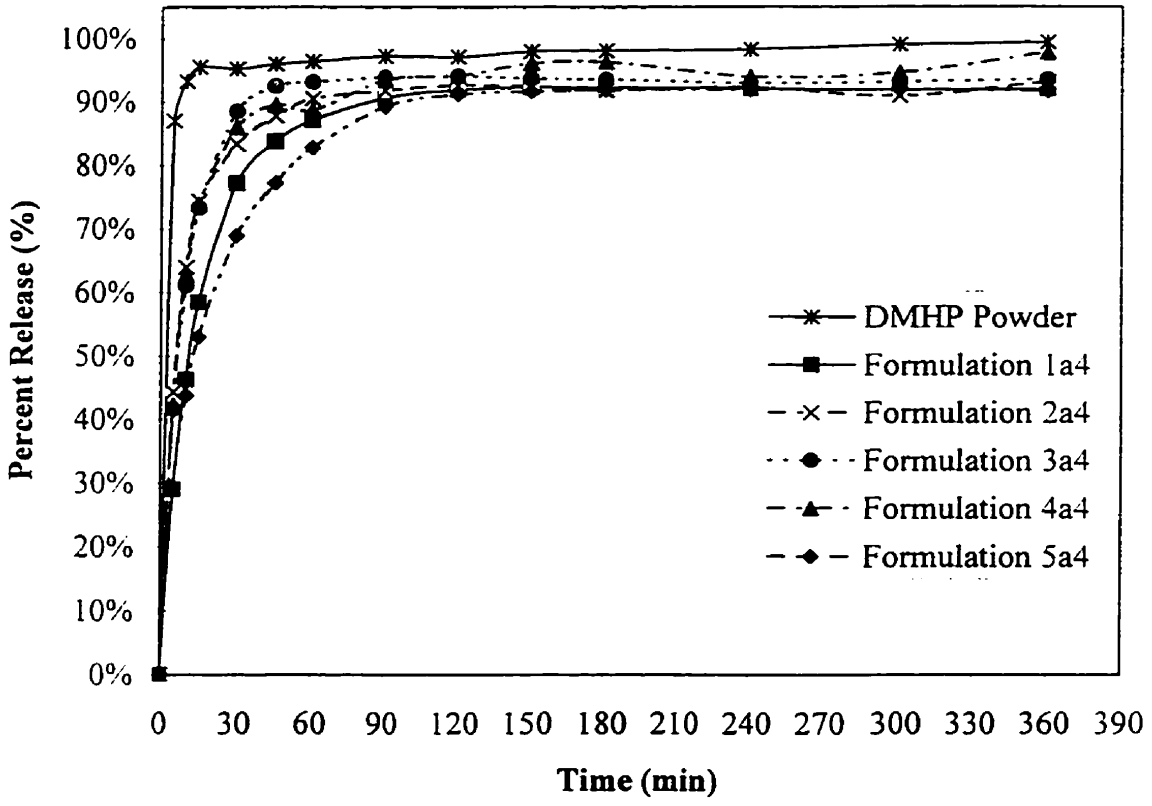


Figure 26: Percent release of DMHP in HCL-KCl pH = 2.0 medium for formulations 1-5. Beads were cured for 15 min. and dried at 40^0C.

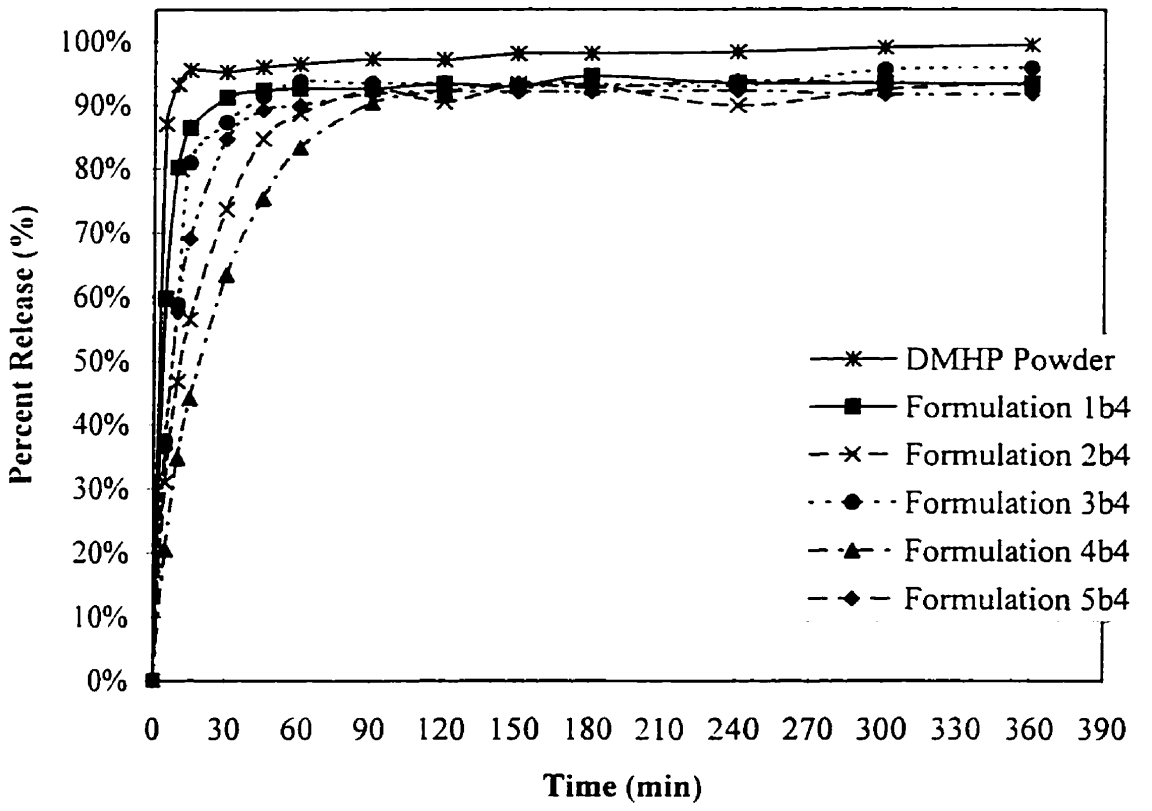


Figure 27: Percent release of DMHP in HCL-KCl pH = 2.0 medium for formulations 1-5. Beads were cured for 30 min. and dried at 40^0C.

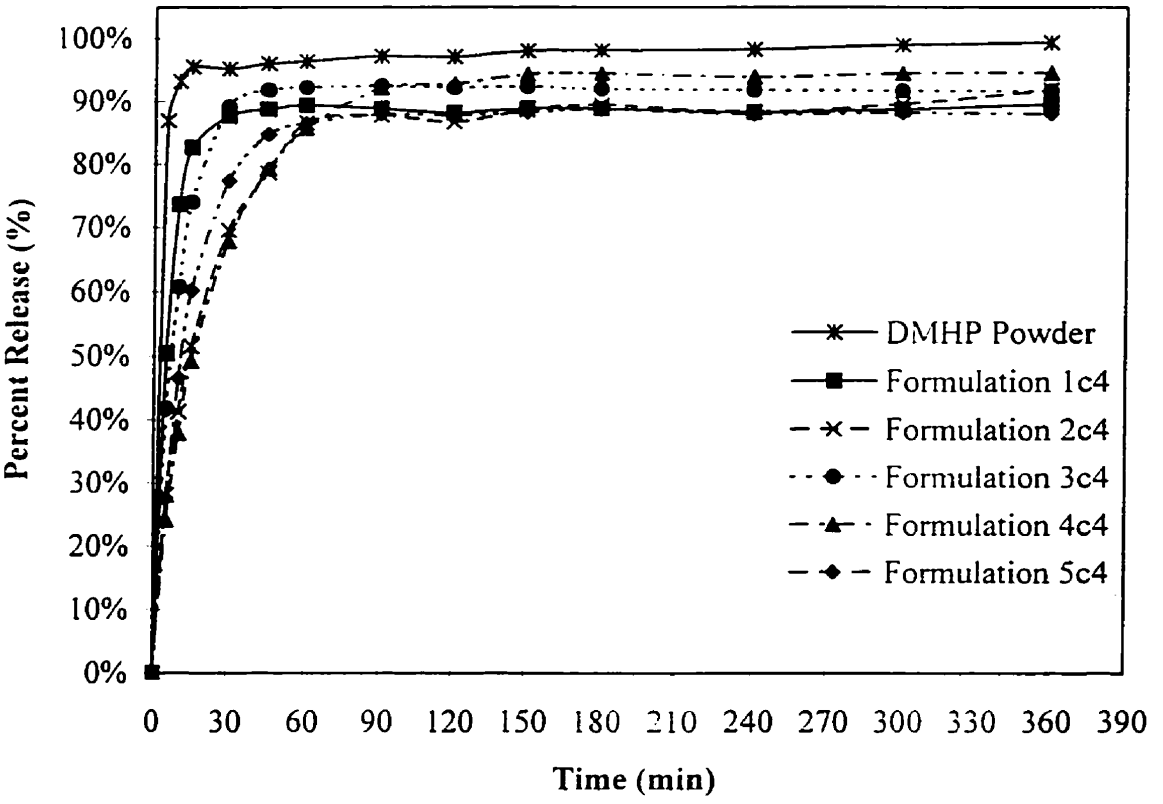


Figure 28: Percent release of DMHP in HCL-KCl pH = 2.0 medium for formulations 1-5. Beads were cured for 45 min. and dried at 40^0C.

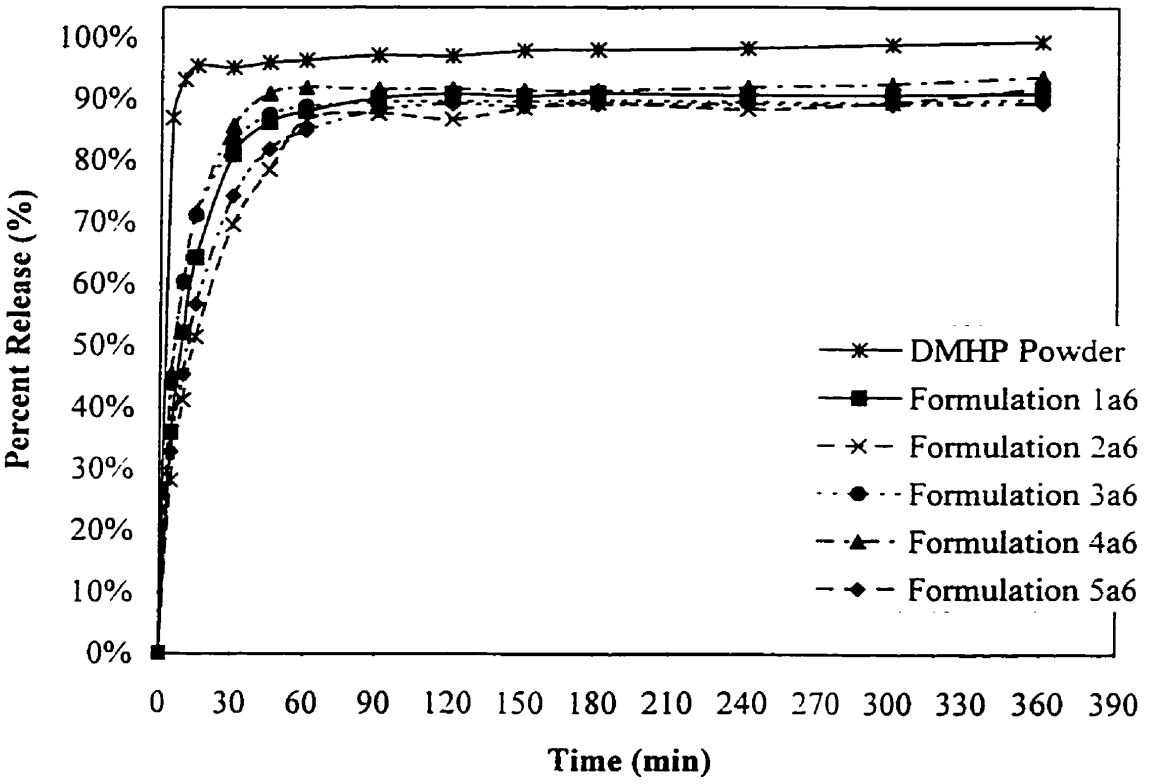


Figure 29: Percent release of DMHP in HCL-KCl pH = 2.0 medium for formulations 1-5. Beads were cured for 15 min. and dried at 60^0C.

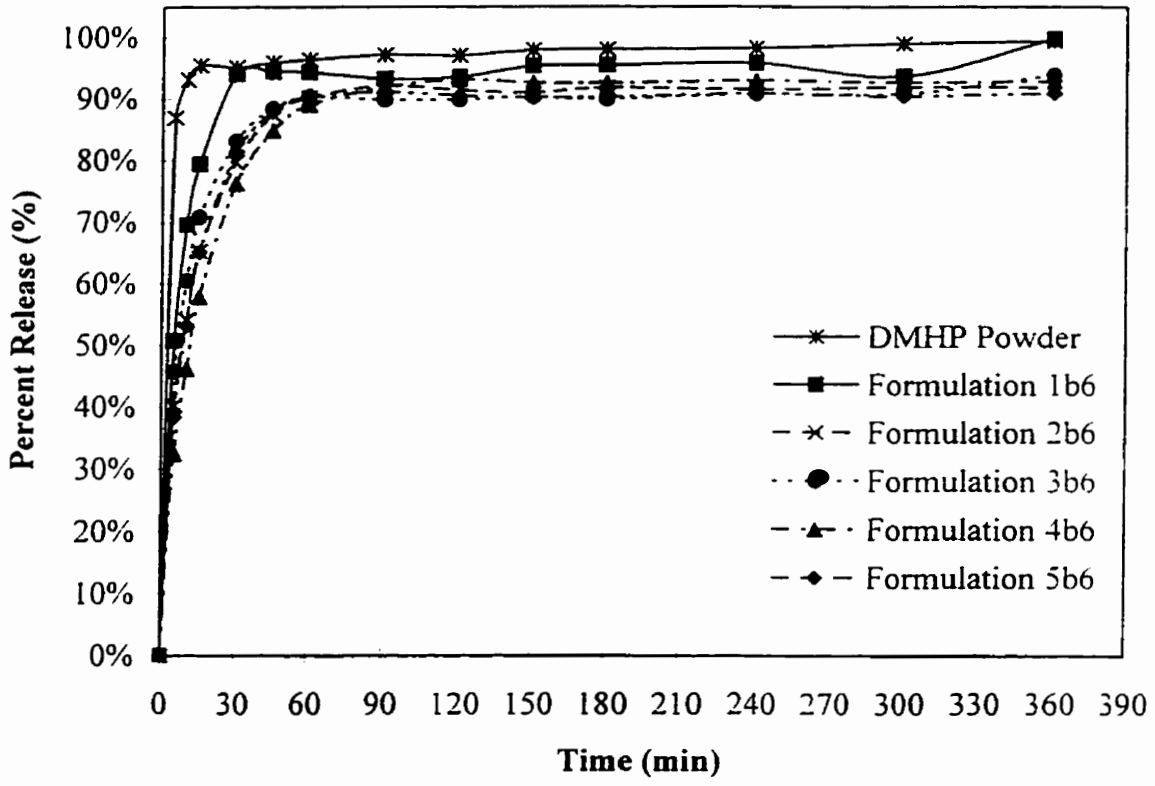


Figure 30: Percent release of DMHP in HCL-KCl pH = 2.0 medium for formulations 1-5. Beads were cured for 30 min. and dried at 60^0C.

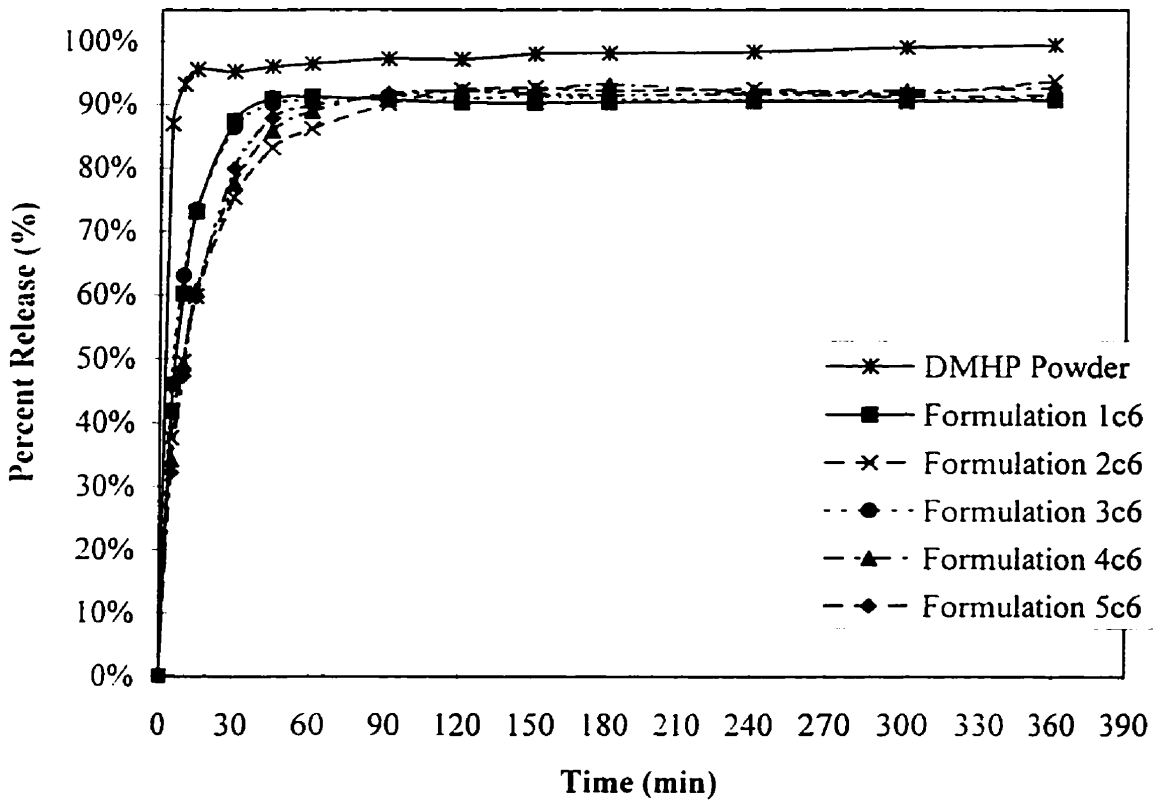


Figure 31: Percent release of DMHP in HCL-KCl pH = 2.0 medium for formulations 1-5. Beads were cured for 45 min. and dried at 60^0C.

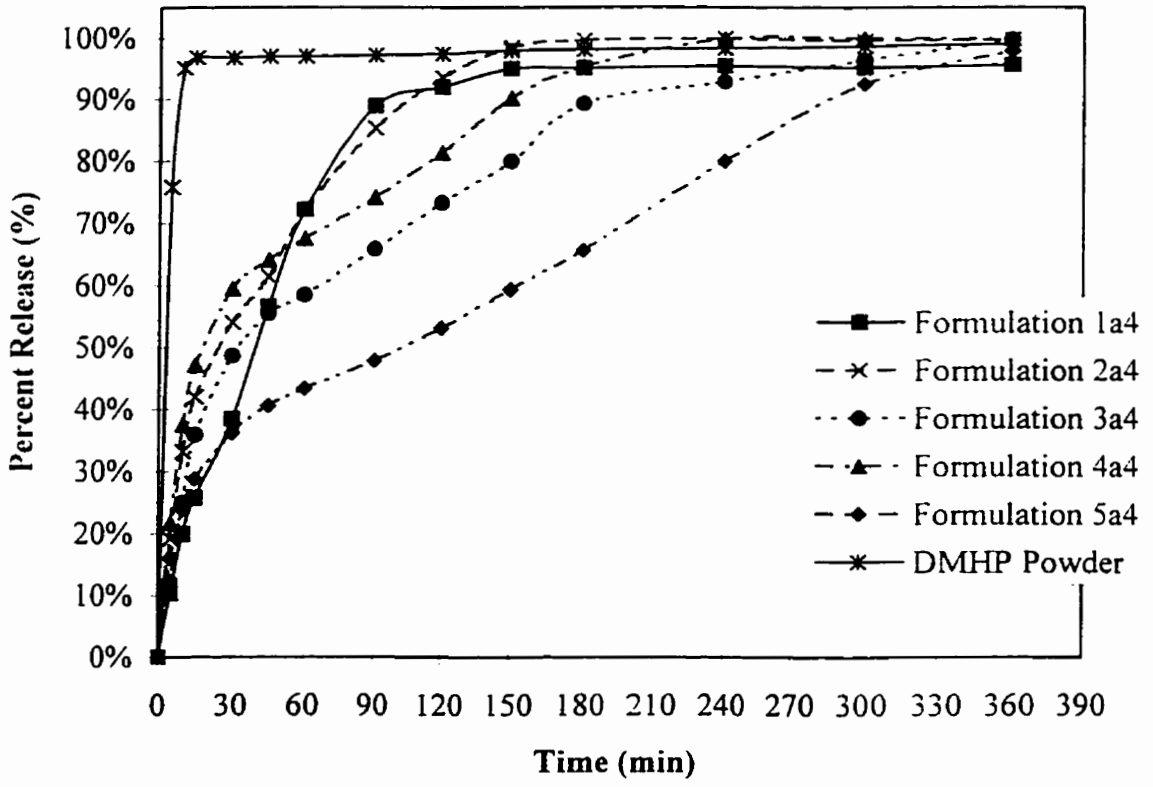


Figure 32: Percent release of DMHP in phosphate pH = 7.4 medium for formulations 1-5. Beads were cured for 15 min. and dried at 40^0C.

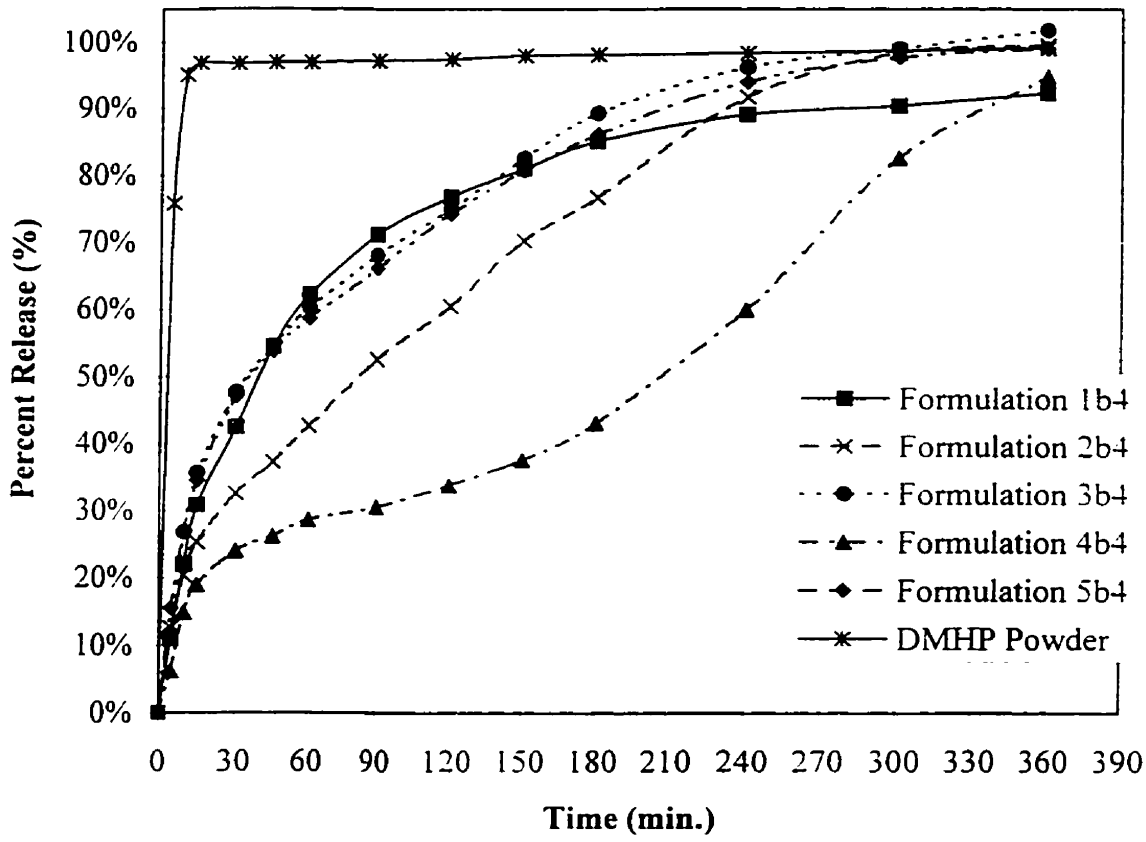


Figure 33: Percent release of DMHP in phosphate pH = 7.4 medium for formulations 1-5. Beads were cured for 30 min. and dried at 40^0C.

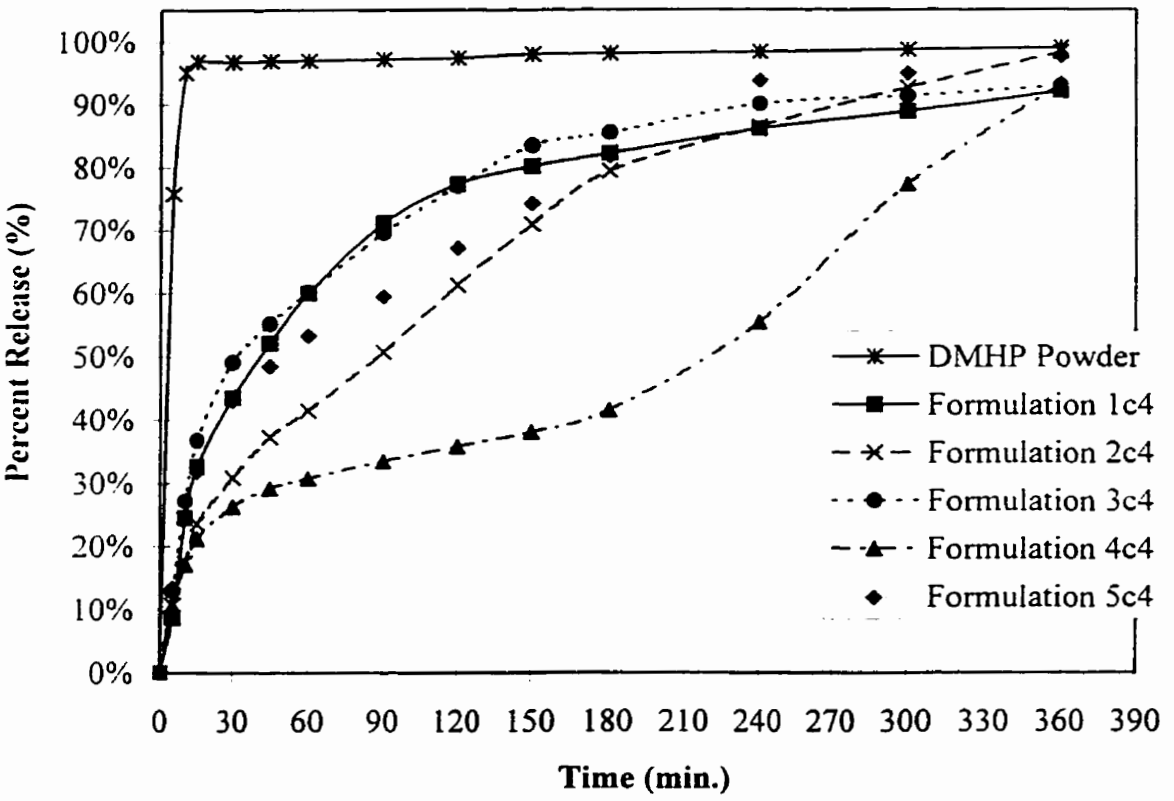


Figure 34: Percent release of DMHP in phosphate pH = 7.4 medium for formulations 1-5. Beads were cured for 45 min. and dried at 40^0C.

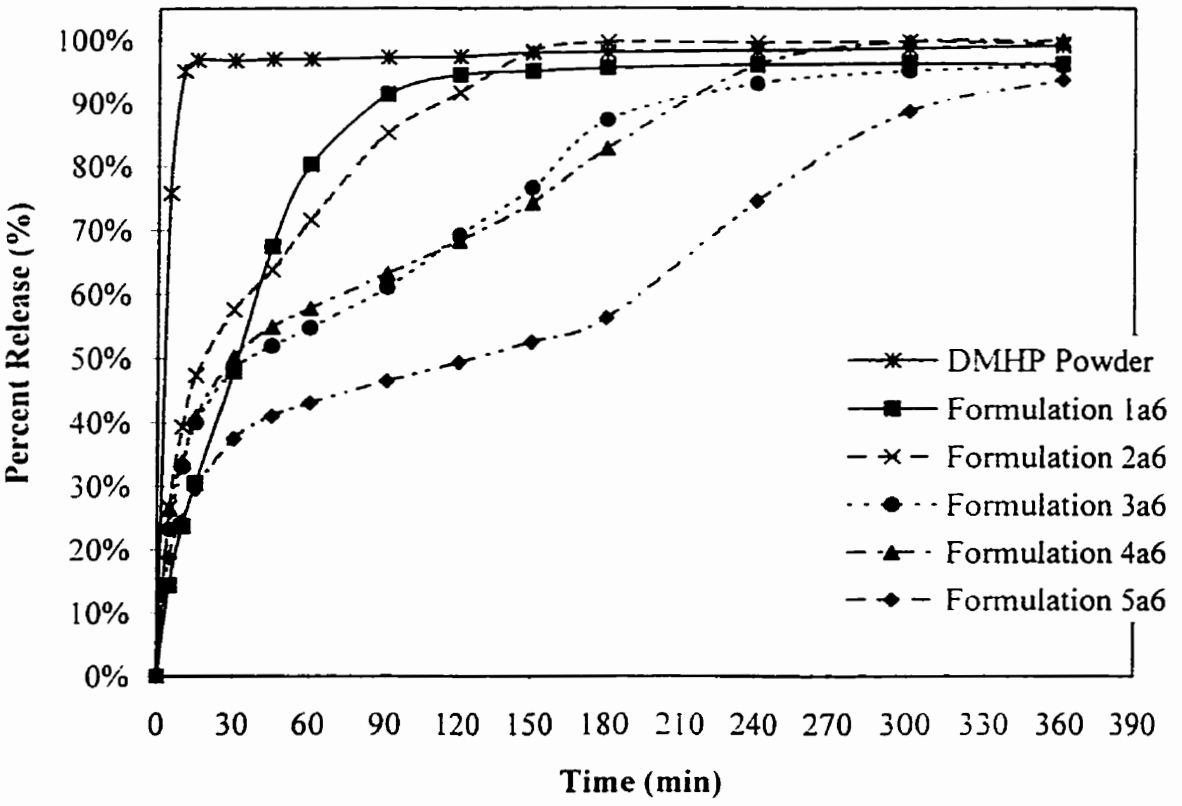


Figure 35: Percent release of DMHP in phosphate pH = 7.4 medium for formulations 1-5. Beads were cured for 15 min. and dried at 60^0C.

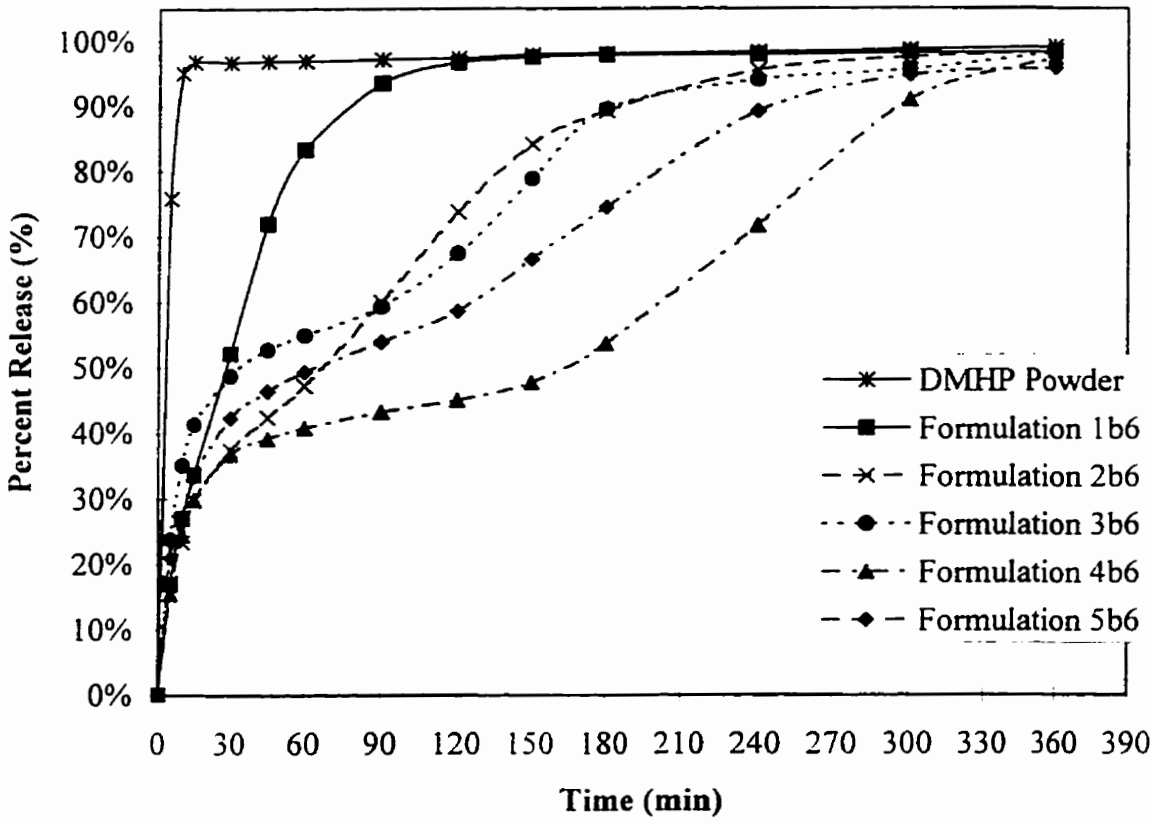


Figure 36: Percent release of DMHP in phosphate pH = 7.4 medium for formulations 1-5. Beads were cured for 30 min. and dried at 60^0C.

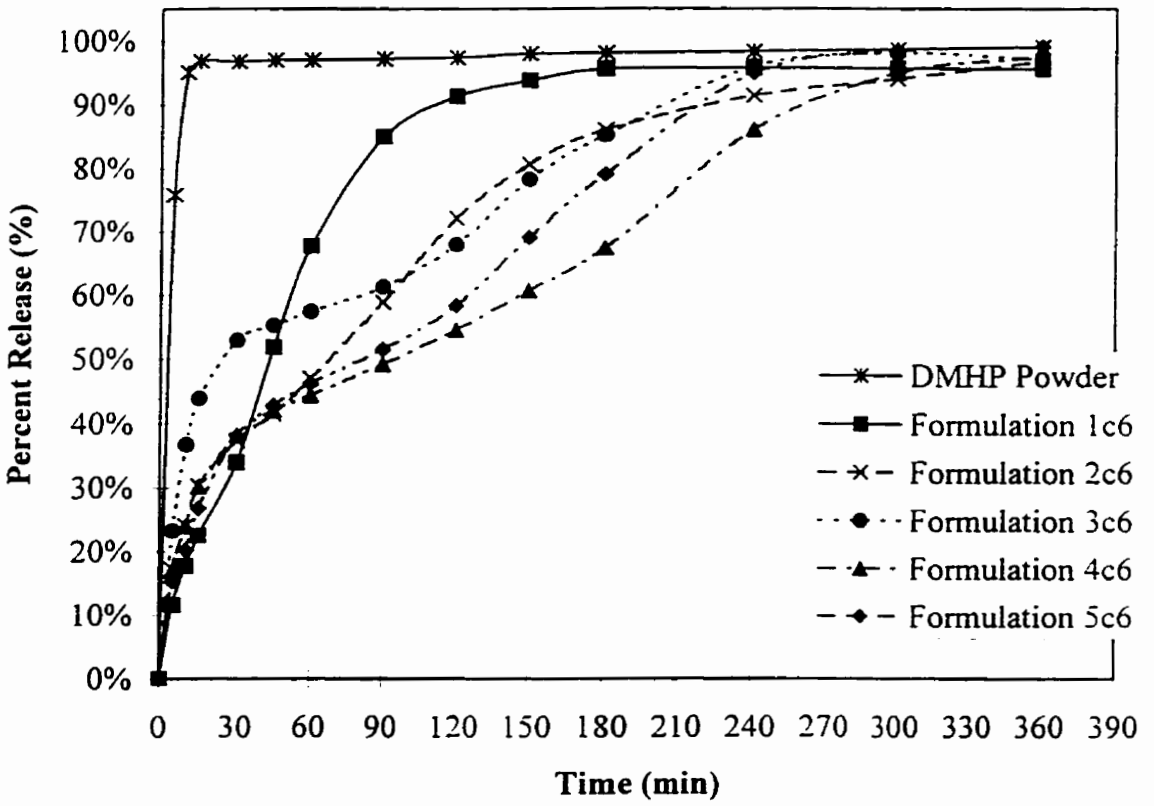


Figure 37: Percent release of DMHP in phosphate pH = 7.4 medium for formulations 1-5. Beads were cured for 45 min. and dried at 60^{0}C.

3.9. DISSOLUTION AFTER ADDITION OF ADDITIVES

Low viscosity grade alginate at a concentration of 3% w/v was utilized for the evaluation of the addition of various other components used in microencapsulation. Alginate alone was unable to inhibit the rate or extent of DMHP release in pH=2.0 KCl-HCl media. Increasing concentrations or increasing the length of curing times did not affect DMHP release (Figure 26-31). The addition of various ingredients into the formulation may help modify the rapid release of DMHP in this medium and also contribute to the modified releasing properties of this system in pH=7.4 phosphate buffer.

Propylene glycol was evaluated as a plasticizer for the alginate. A plasticizer may cause the alginate strands to align more uniformly in the lattice structure which could help reduce the development of pores and cracks. Figures 38 and 39 show the release of DMHP from calcium alginate beads in pH=2.0 and pH=7.4 media that have propylene glycol added. The release of DMHP at pH=2.0 approaches 100% after the first 30 min. compared to formulations without propylene glycol (Formulations 4b4 and 4b6) which have a similar release profile, signifying that the release was not affected by propylene glycol (Figure 38). DMHP release from beads containing propylene glycol in phosphate media pH=7.4 was similar for the two drying temperatures and not delayed when compared to the release from 3% alginate alone (Figure 39). Formulations 11a and 11b were prepared with 20 mL of propylene glycol. The addition of more propylene glycol was not possible as the alginate solution became too viscous for efficient dripping. The amount of propylene glycol used was based on total volume of the polymer, so 10% v/v was added. Since this amount is high when compared to the polymer weight, and because

it is a small water miscible molecule, minimal plasticization may have occurred. Further work needs to be done to evaluate the role of propylene glycol.

Hydroxypropylmethyl cellulose (HPMC) will swell, independent of pH, in the presence of aqueous medium. It was hypothesized that when the dried beads containing HPMC are exposed to aqueous medium pH=2.0, the HPMC will swell and prevent the leaching of DMHP. The beads had to be specially formulated to accommodate the HPMC component (Page 63). Limitations on the concentration and grade of HPMC that could be used were restricted by an increase in the viscosity of the solution. This prevented the solution from flowing through the dripping apparatus. A reduction in the alginate concentration to 2 % w/v (Formulations 9a - 9d) was used to reduce the viscosity. Formulations 10a and 10b containing 3% w/v alginate, was the maximum alginate concentration that could be formulated with HPMC. In Figures 40 and 41 is shown the release of DMHP in pH=2.0 and pH=7.4 medium respectively. Here also the release of DMHP could not be delayed by the addition of HPMC. Although the maximum amount released does seem to be reduced, with approximately 80% release after 30 min for some formulations the rate of release is not appreciably delayed (Figure 40). Release in pH=7.4 phosphate buffer (Figure 41) was similar for all 6 formulations except for formulation 10b which was similar to the release of 3% alginate (Formulation 4b6) without additives (Figure 39).

Pectin is composed primarily of α-D-galactopyranosyluronic acid units. Treatment with sodium hydroxide de-esterifies the molecule to produce ionizable sodium pectate that can undergo ionotropic gelation. In contrast to alginate, pectin's interaction with cations is relatively weak. The addition of pectin in the sodium alginate solution

was proposed to possibly add some additional structure to seal the pores and cracks, and reduce the rate of DMHP release in pH=2.0 media. The release is displayed in Figures 42 and 43 at pH=2.0 and pH=7.4 respectively. The incorporation of pectin appeared to have no effect on the rate of release of DMHP in pH=2.0 which approached 100% by 60 min. (Figure 42). In pH=7.4 phosphate buffer the release was relatively rapid within the first 45 min. This probably results during the initial hydration and swelling of the beads. After the first 45 min. the release rate follows a zero order pattern for all formulations irrespective of pectin concentration and drying temperatures (Figure 43).

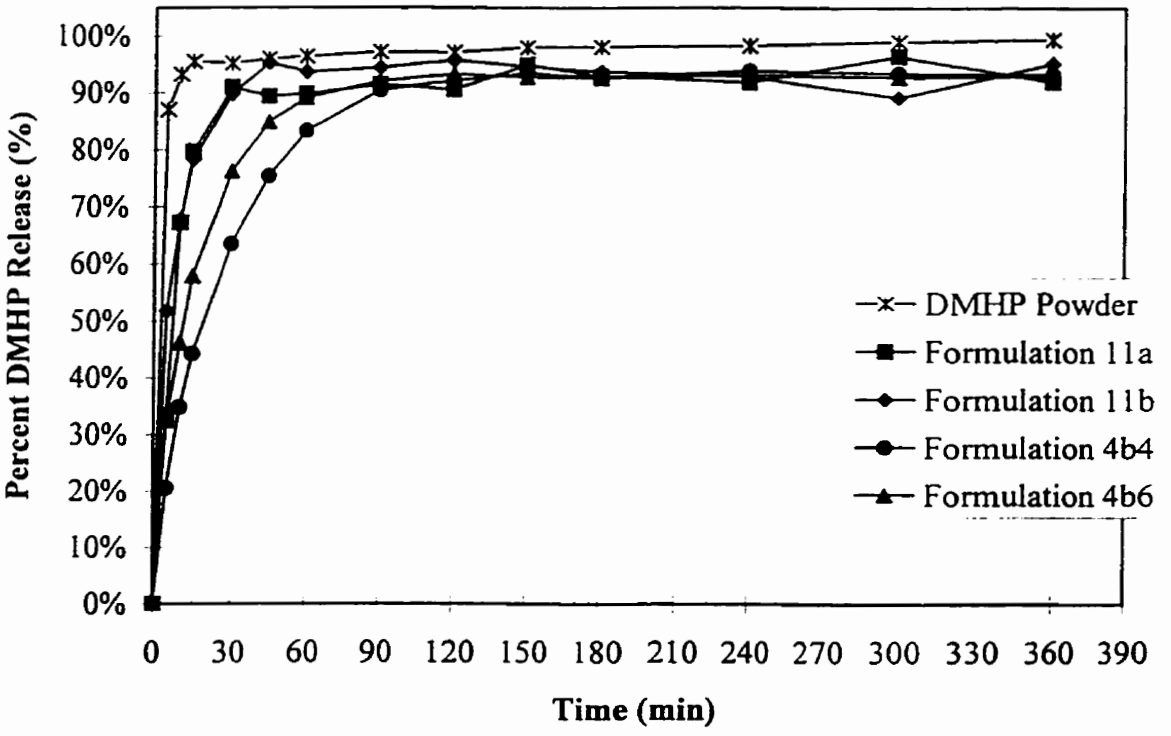


Figure 38: Release of DMHP from 3% w/v alginate beads plasticized with propylene glycol in pH = 2.0 KCl-HCl buffer cured for 30 min. and dried at 40^{0}C (11a) and dried at 60^{0}C (11b). Also shown is 3% alginate beads with no plasticizer cured 30 min. and dried at 40^{0}C (4b4) and dried at 60^{0}C (4b6).

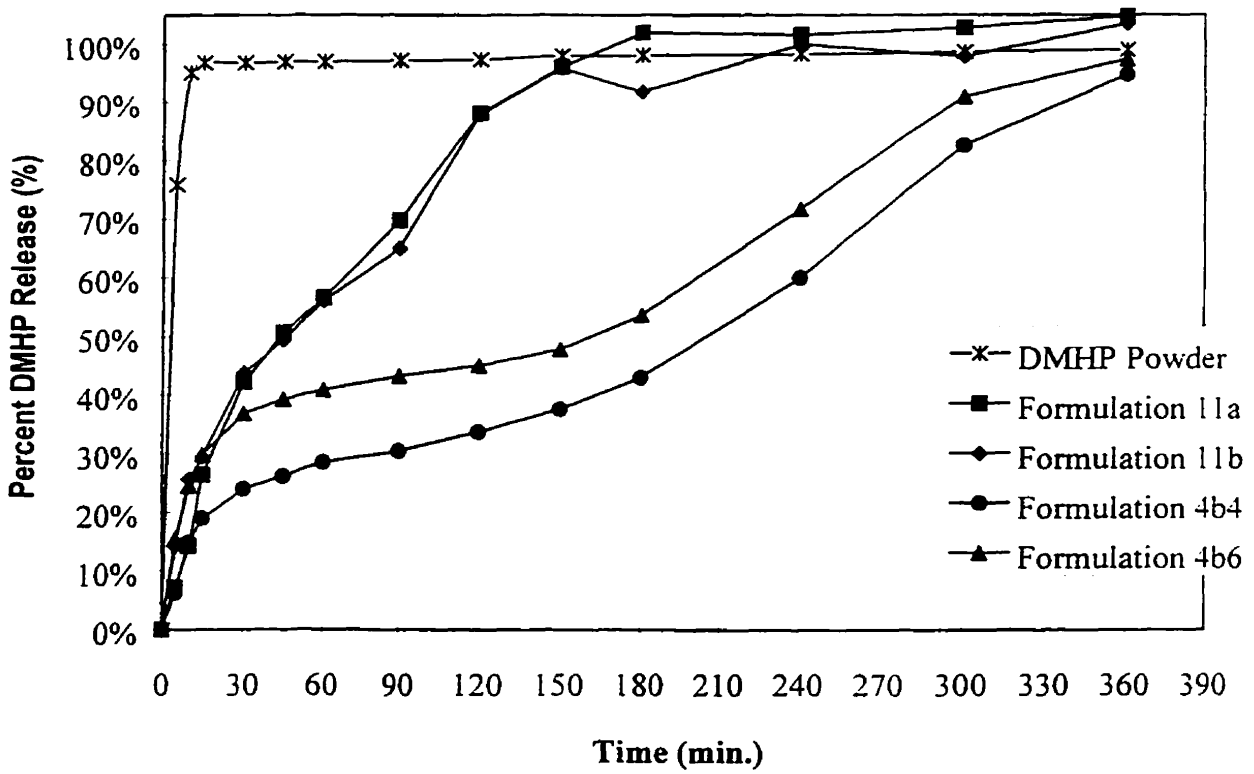


Figure 39: Release of DMHP from 3% w/v alginate beads plasticized with propylene glycol in pH = 7.4 phosphate buffer cured for 30 min. and dried at 40^{0}C (11a) and dried at 60^{0}C (11b). Also shown is 3% alginate beads with no plasticizer cured 30 min. and dried at 40^{0}C (4b4) and dried at 60^{0}C (4b6).

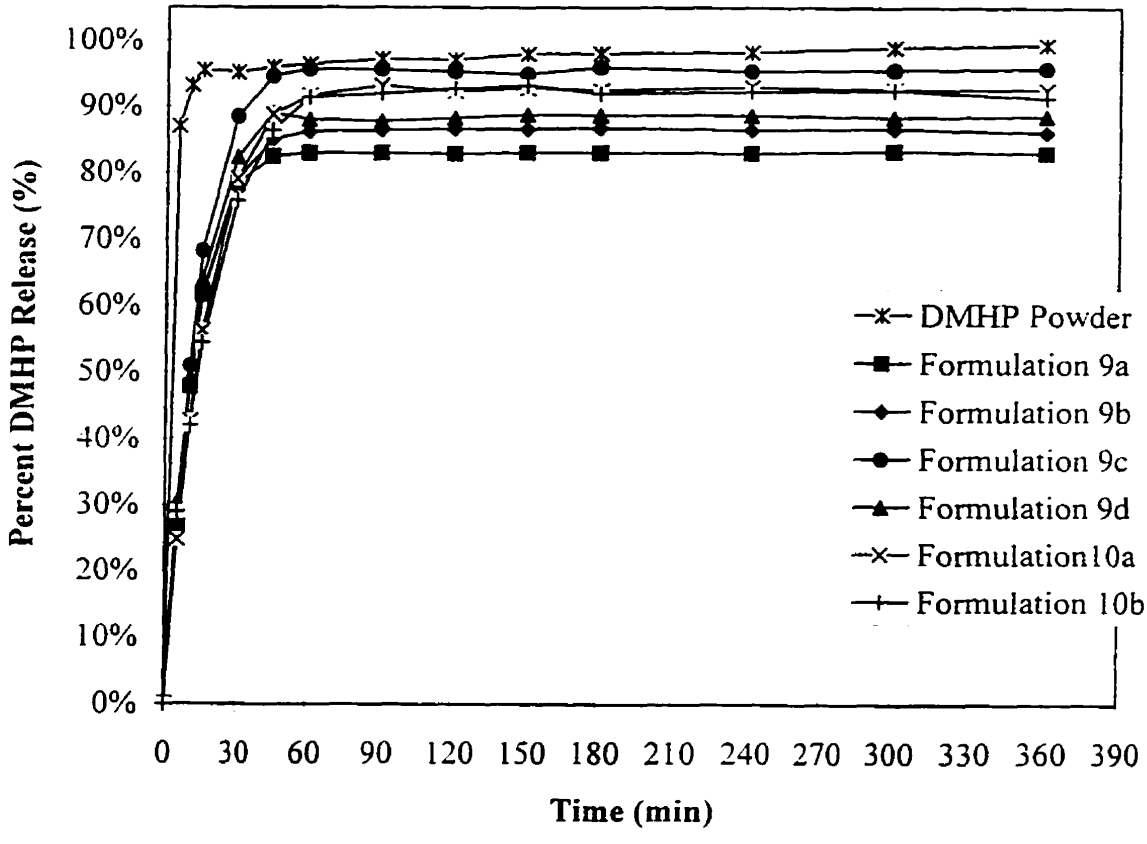


Figure 40: Release of DMHP from 2% alginate beads with 0.5% HPMC cured for 30 min. and dried at 40^{0}C (9a) and dried at 60^{0}C (9b) also cured for 60 min. with drying at 40^{0}C (9c) and drying at 60^{0}C (9d) in pH = 2.0 KCl-HCl buffer. Formulations 10a and 10b are 3% alginate beads cured for 30 min. and dried at 40^{0}C and 60^{0}C respectively.

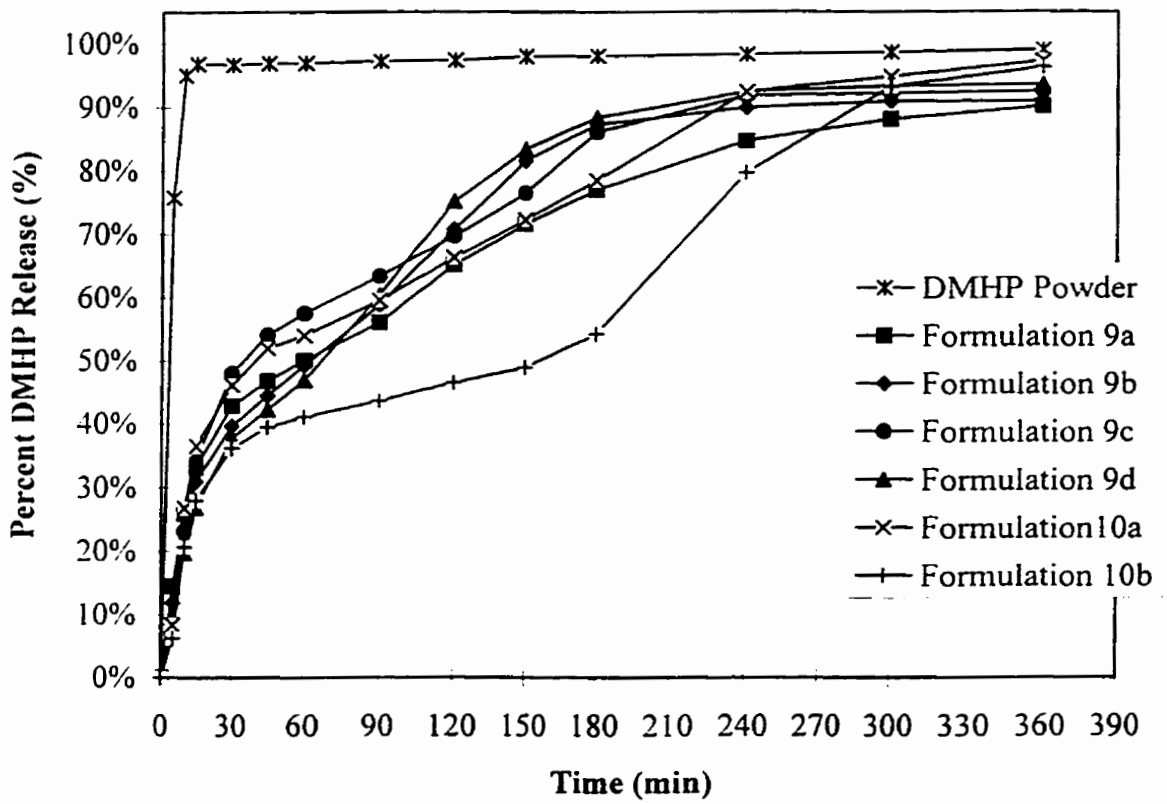


Figure 41: Release of DMHP from 2% alginate beads with 0.5% HPMC cured for 30 min. and dried at 40°C (9a) and dried at 60°C (9b) also cured for 60 min. with drying at 40°C (9c) and drying at 60°C (9d) in pH = 7.4 phosphate buffer. Formulations 10a and 10b are 3% alginate beads cured for 30 min. and dried at 40°C and 60°C respectively.

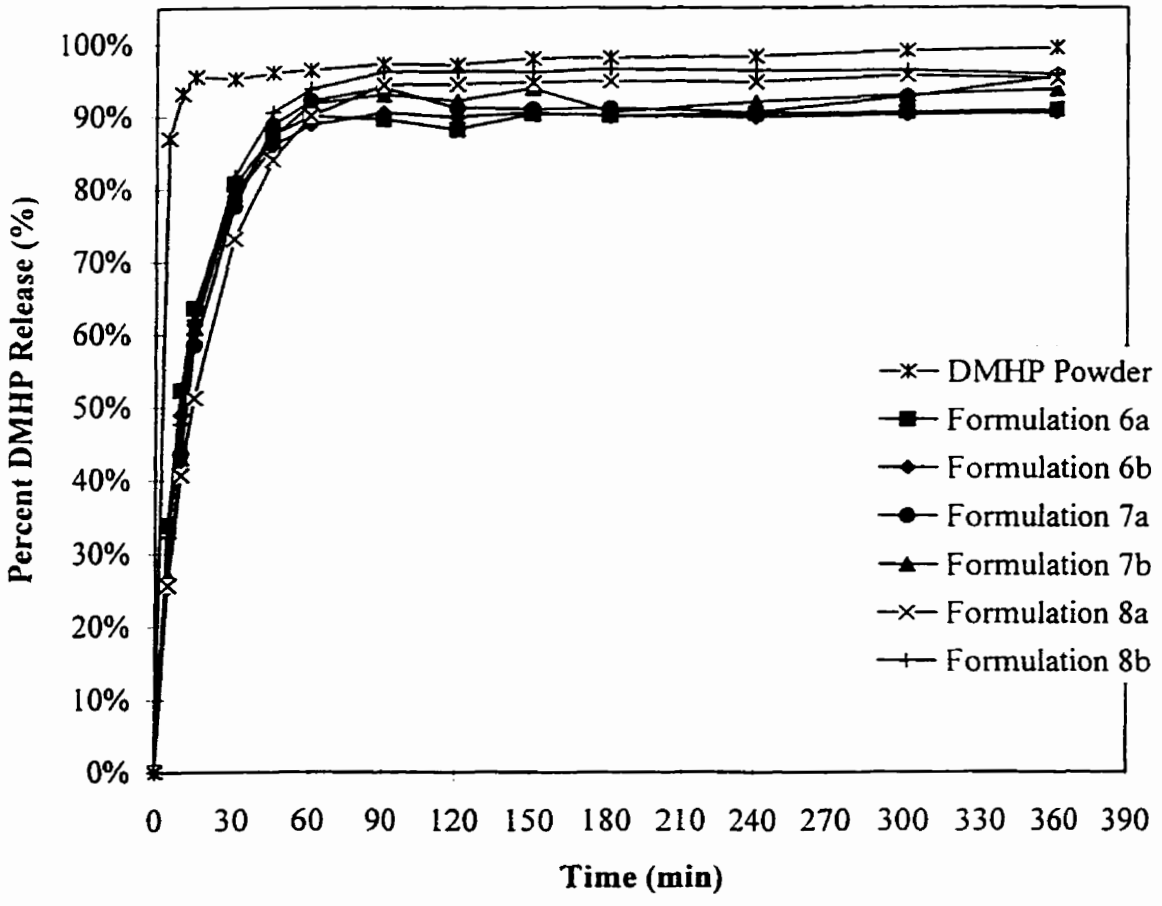


Figure 42: Release of DMHP from calcium alginate-pectin beads in pH = 2.0 KCl-HCl buffer. All formulations were made with 3% alginate with 1%, 2%, or 3% pectin (formulations 6, 7, and 8 respectively). All cured for 30 min. and dried at either 40^{0}C (6a, 7a, and 8a) or 60^{0}C (6b, 7b, and 8b).

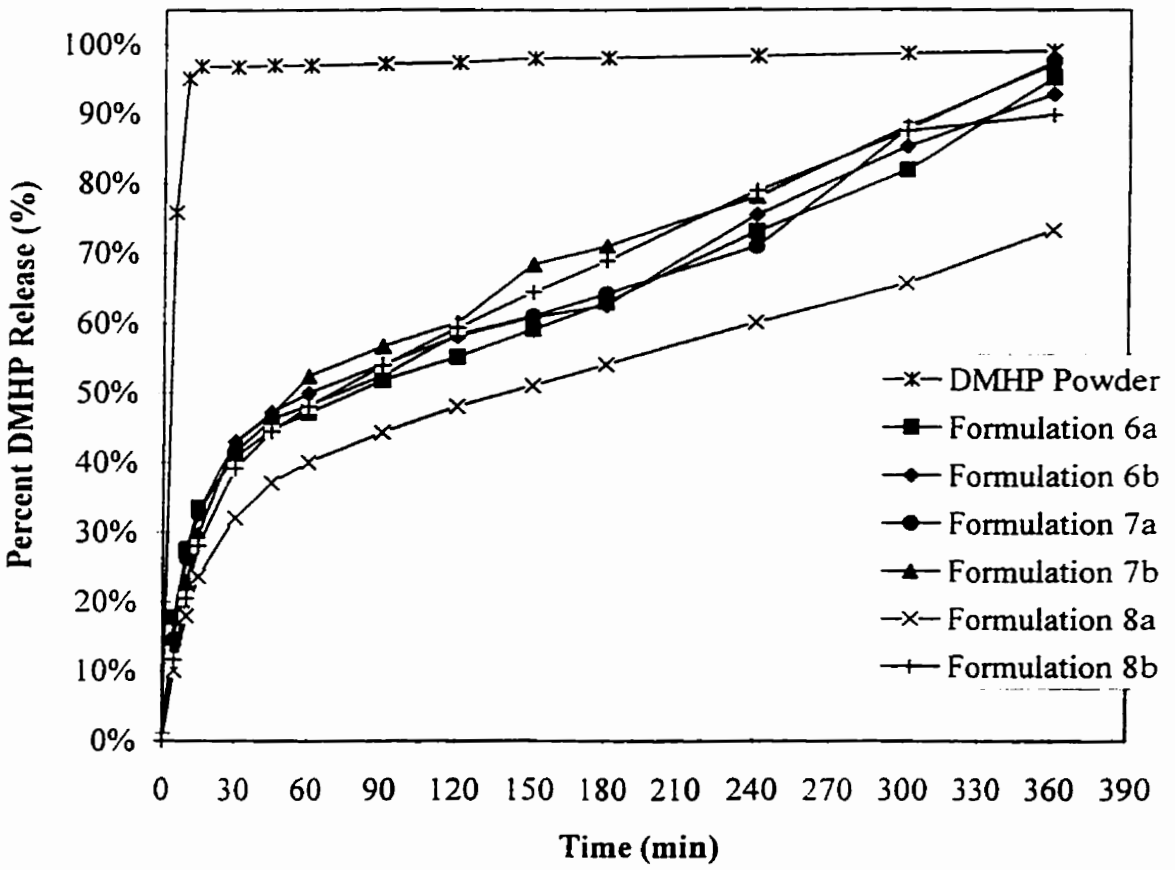


Figure 43: Release of DMHP from calcium alginate-pectin beads in pH = 7.4 phosphate buffer. All formulations were made with 3% alginate with 1%, 2%, or 3% pectin (formulations 6, 7, and 8 respectively). All cured for 30 min. and dried at either 40^0C (6a, 7a, and 8a) or 60^0C (6b, 7b, and 8b).

3.10. DATA ANALYSIS

The rate of DMHP release from calcium alginate beads was compared statistically to pure DMHP at the 2 hour time point during dissolution. Comparisons were done to evaluate differences in alginate concentration, curing time, and drying temperature. One way analysis of variance (ANOVA) was used to test the null hypothesis that there is no difference among the populations from which the samples were drawn or that there is no difference in release of DMHP from beads or powder with the three different production parameters tested. Further paired comparisons using Student-Newman-Keuls (SNK) multiple comparison test were done to isolate differences in the experimental group. The comparison was done using a confidence interval of 95% or $P>0.05$. All analysis was performed using Jandel Scientific SigmaStat v2.0 software. The abbreviations used on the data output by the software are as follows:

Std Dev - Standard Deviation
SEM - Standard error of the mean
DF - Degrees of Freedom
SS - Sum of squares
MS - Mean of squares
F - The test statistic
P - Probability that the differences may be due to chance at a certain confidence level

Mathematical treatment of formulations 6-8 were done to evaluate the drug release mechanism. The zero order release model was used to describe the percent release versus time. The equation given by integration of Equation 6 from time t to ∞ is:

$$\frac{M_t}{M_\infty} = k_m t$$

where M_t and M_∞ are the amount of drug release at time t and ∞ respectively. The plot should yield a straight line with slope k_m. The assumption was made that DMHP release would always achieve 100% even though release was only evaluated up to 6 h and was not always complete by that time.

The square root of time drug release model (Equations 17 and 18) was proposed by Higuchi to describe the release in a matrix system.[59, 60] A plot of drug release vs. the square root of time should be linear if the release is matrix diffusion controlled.

The ANOVA and SNK data for formulations 1-5 are shown in Appendices 1-6 displays. There is a statistical significant differences between the release of DMHP at pH=7.4 from the calcium alginate beads as compared to DMHP powder. The SNK multiple comparisons of the calcium alginate beads are shown in appendices 1-7. There was no significant difference in release when the beads were dried at 40^{0}C or 60^{0}C irrespective of alginate concentration or curing time. For each curing time, only 3% alginate showed significant differences from times 15 – 30 and 15 – 45 min. Using alginate at a concentration of 3%, the largest difference in release was shown at curing times of 30 and 45 min.

Table IX displays the regression analysis of formulations 6-8 with the data set starting at the 45 min. sample, the point when the curve becomes linear. The region from zero to 45 min. is the time of immediate release while the beads are hydrating and swelling. All formulations resulted in a good correlation with the R^2 values ranging from 0.9788 – 0.9945. The square root of time plot is shown in Figure 44 and the linear regression data of the plots is displayed in Table X. The formulations again had good correlation to the Higuchi model for matrix diffusion with R^2 values ranging from 0.9412

– 0.9938. The release of DMHP from the pectin-alginate microspheres is a complex process. Since there is good correlation with the Higuchi model for a non-disentigrating matrix, the main release mechanism appears to be diffusion controlled even though there is some bead erosion. Thus the erosion of the beads appears to be an insignificant component of DMHP release, but for a larger molecule, erosion may become a more dominant factor than diffusion.

Table IX: Linear regression data on Formulation 6-8.

	Y=MX+B		
Formulation	M	Y-Intercept	R^2
6a	0.0015	0.37	0.9927
6b	0.0015	0.4024	0.9899
7a	0.0016	0.3788	0.9812
7b	0.0015	0.4255	0.9918
8a	0.0011	0.3388	0.9945
8b	0.0015	0.4044	0.9788

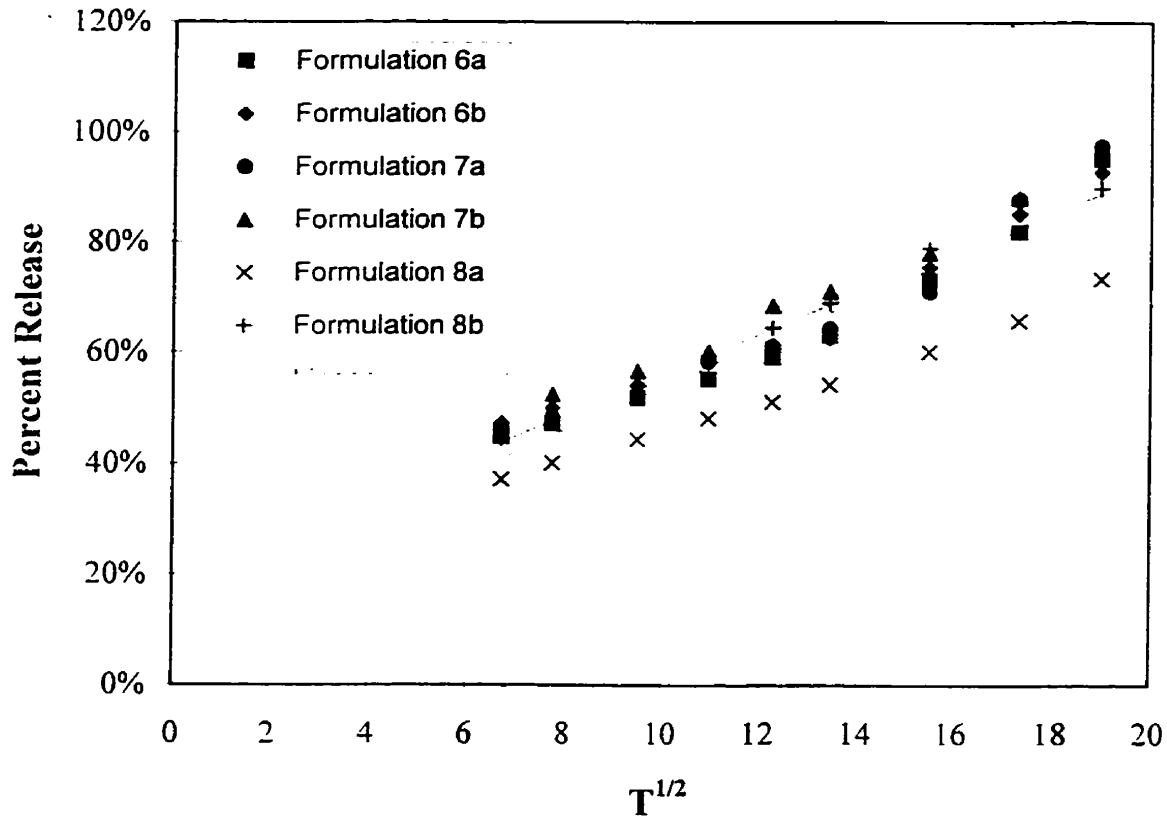


Figure 44: Square root of time vs. percent release of DMHP for calcium alginate-pectin formulations.

Table X: Linear regression data for Higuchi equation analysis of Figure 44.

	Y=MX+B		
Formulation	M	Y-Intercept	R^2
6A	0.0391	0.147	0.9563
6B	0.0369	0.192	0.9572
7A	0.0402	0.149	0.9412
7B	0.0393	0.197	0.9865
8A	0.0282	0.174	0.9909
8B	0.0390	0.175	0.9938

CHAPTER IV

CONCLUSION

These studies were designed to evaluate the hypothesis that calcium alginate beads could be used to prepare a modified release dosage form of DMHP. Calcium alginate beads incorporating DMHP were prepared using a parastalic pump apparatus to facilitate controlled flow addition (dropwise) of the alginate-DMHP solution into a curing solution containing calcium chloride. Higher concentrations of sodium alginate resulted in higher encapsulation efficiency and more uniform bead size distribution. The release of DMHP was complete from all calcium alginate beads regardless of formulation within 45 min. in pH=2.0 media simulating gastric fluid. This limits the use of calcium alginate beads to prolong DMHP's release. The release was not reduced when the alginate concentration or the curing time was increased.

Dissolution studies in pH=7.4 media were conducted based on the assumption that there was minimal DMHP release in the gastric environment at pH=2.0. This could be accomplished by enteric coating of the beads. This approach was not evaluated in the current series of experiments. The release profile in pH=7.4 media demonstrated an initial hydration and swelling of the beads which allows DMHP to diffuse out rapidly. This initial burst last for 30-45 min., followed by a prolonged release as the beads slowly

erode by a β-elimination reaction.[82, 83] The use of higher concentrations of sodium alginate provides a rate of release that yields a concentration versus time profile that can be described by a sigmodial shaped curve. Drying the beads at different temperatures does not effect the rate of release significantly, and thus the degree of bead hydration after drying is insignificant.

To help retard the rapid release of DMHP in the pH=2.0 medium, additives such as propylene glycol, HMPC, or pectin were added to the formulation for evaluation. The addition of these compounds did not effectively minimize the extensive release in pH=2.0 media, but formulations containing HPMC seemed to have a reduction in the total amount of DMHP released.

Pectin-alginate combination formulations showed the most promising profile for release of DMHP in pH=7.4 medium. The ability of pectin to also undergo ionotropic gelation results in a DMHP release rate which correlates well with the zero order release model (R^2 = 0.9945) and with the Higuchi model for matrix diffusion release (R^2 = 0.9938).

The release of DMHP a low molecular weight, water soluble compound was sufficiently prolonged using formulations with high sodium alginate or when alginate is combined with pectin in pH=7.4 medium. The major limitation is the complete release in acid medium, which would be first seen when administered *in vivo*. Methods such as coating of the beads directly or coating a delivery form such as a tablet or capsule that encases the beads with a enteric polymer may reduce or prevent this immediate release.

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Appendix 1: ANOVA and SNK at 2 hour release in phosphate pH=7.4 buffer for formulations 1-5 cured for 15 min and dried at 40^0C

Normality Test: Passed (P = 0.071)

Equal Variance Test: Passed (P = 0.101)

Group	Mean	Std Dev	SEM
DMHP Powder	97.400	1.345	0.777
1% Alginate	91.967	4.428	2.556
2% Alginate	93.533	4.535	2.618
2.5% Alginate	73.267	9.124	5.268
3% Alginate	81.367	15.842	9.146
3.5% Alginate	53.200	17.229	9.947

Power of performed test with alpha = 0.050: 0.957

Source of Variation	DF	SS	MS	F	P
Between Treatments	5	4126.071	825.214	7.357	0.002
Residual	12	1346.047	112.171		
Total	17	5472.118			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.002).

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparison	Diff of Means	p	q	P<0.05
DMHP Powder vs. 3.5% Alginate	44.200	6	7.228	Yes
DMHP Powder vs. 2.5% Alginate	24.133	5	3.947	No
DMHP Powder vs. 3% Alginate	16.033	4	2.622	No
DMHP Powder vs. 1% Alginate	5.433	3	0.889	No
DMHP Powder vs. 2% Alginate	3.867	2	0.632	No
2% Alginate vs. 3.5% Alginate	40.333	5	6.596	Yes
2% Alginate vs. 2.5% Alginate	20.267	4	3.314	No
2% Alginate vs. 3% Alginate	12.167	3	1.990	No
2% Alginate vs. 1% Alginate	1.567	2	0.256	No
1% Alginate vs. 3.5% Alginate	38.767	4	6.340	Yes
1% Alginate vs. 2.5% Alginate	18.700	3	3.058	No
1% Alginate vs. 3% Alginate	10.600	2	1.734	No
3% Alginate vs. 3.5% Alginate	28.167	3	4.606	Yes
3% Alginate vs. 2.5% Alginate	8.100	2	1.325	No
2.5% Alginate vs. 3.5% Alginate	20.067	2	3.282	Yes

Appendix 2: ANOVA and SNK at 2 hour release in phosphate pH=7.4 buffer for formulations 1-5 cured for 30 min and dried at 40^0C

Normality Test: Passed (P = 0.100)

Equal Variance Test: Passed (P = 0.093)

Group	Mean	Std Dev	SEM
DMHP Powder	97.400	1.345	0.777
1% Alginate	76.767	12.352	7.131
2% Alginate	60.525	30.949	15.475
2.5% Alginate	75.000	11.505	6.643
3% Alginate	33.833	10.130	5.848
3.5% Alginate	74.333	3.620	2.090

Power of performed test with alpha = 0.050: 0.795

Source of Variation	DF	SS	MS	F	P
Between Treatments	5	6790.857	1358.171	4.800	0.010
Residual	13	3678.448	282.958		
Total	18	10469.304			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.010).

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparison	Diff of Means	p	q	P<0.05
DMHP Powder vs. 3% Alginate	63.567	6	6.545	Yes
DMHP Powder vs. 2% Alginate	36.875	5	4.059	No
DMHP Powder vs. 3.5% Alginate	23.067	4	2.375	No
DMHP Powder vs. 2.5% Alginate	22.400	3	2.306	No
DMHP Powder vs. 1% Alginate	20.633	2	2.125	No
1% Alginate vs. 3% Alginate	42.933	5	4.421	No
1% Alginate vs. 2% Alginate	16.242	4	1.788	No
1% Alginate vs. 3.5% Alginate	2.433	3	0.251	No
1% Alginate vs. 2.5% Alginate	1.767	2	0.182	No
2.5% Alginate vs. 3% Alginate	41.167	4	4.239	Yes
2.5% Alginate vs. 2% Alginate	14.475	3	1.593	No
2.5% Alginate vs. 3.5% Alginate	0.667	2	0.0686	No
3.5% Alginate vs. 3% Alginate	40.500	3	4.170	Yes
3.5% Alginate vs. 2% Alginate	13.808	2	1.520	No
2% Alginate vs. 3% Alginate	26.692	2	2.938	No

Appendix 3: ANOVA and SNK at 2 hour release in phosphate pH=7.4 buffer for formulations 1-5 cured for 45 min and dried at 40^0C

Normality Test: Passed (P = 0.450)

Equal Variance Test: Passed (P = 0.518)

Group	Mean	Std Dev	SEM
DMHP Powder	97.400	1.345	0.777
1% Alginate	77.433	6.352	3.667
2% Alginate	61.267	14.523	8.385
2.5% Alginate	77.067	12.013	6.935
3% Alginate	35.800	8.029	4.636
3.5% Alginate	67.200	4.101	2.900

Power of performed test with alpha = 0.050: 1.000

Source of Variation	DF	SS	MS	F	P
Between Treatments	5	6316.818	1263.364	14.776	<0.001
Residual	11	940.480	85.498		
Total	6	7257.298			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparison	Diff of Means	p	q	P<0.05
DMHP Powder vs. 3% Alginate	61.600	6	11.539	Yes
DMHP Powder vs. 2% Alginate	36.133	5	6.768	Yes
DMHP Powder vs. 3.5% Alginate	30.200	4	5.060	Yes
DMHP Powder vs. 2.5% Alginate	20.333	3	3.809	No
DMHP Powder vs. 1% Alginate	19.967	2	3.740	Yes
1% Alginate vs. 3% Alginate	41.633	5	7.799	Yes
1% Alginate vs. 2% Alginate	16.167	4	3.028	No
1% Alginate vs. 3.5% Alginate	10.233	3	1.715	No
1% Alginate vs. 2.5% Alginate	0.367	2	0.0687	No
2.5% Alginate vs. 3% Alginate	41.267	4	7.730	Yes
2.5% Alginate vs. 2% Alginate	15.800	3	2.960	No
2.5% Alginate vs. 3.5% Alginate	9.867	2	1.653	No
3.5% Alginate vs. 3% Alginate	31.400	3	5.261	Yes
3.5% Alginate vs. 2% Alginate	5.933	2	0.994	No
2% Alginate vs. 3% Alginate	25.467	2	4.770	Yes

Appendix 4: ANOVA and SNK at 2 hour release in phosphate pH=7.4 buffer for formulations 1-5 cured for 15 min and dried at 60^0C

Normality Test: Passed (P = 0.128)

Equal Variance Test: Passed (P = 0.078)

Group	Mean	Std Dev	SEM
DMHP Powder	97.400	1.345	0.777
1% Alginate	94.467	1.601	0.924
2% Alginate	91.733	5.221	3.015
2.5% Alginate	69.167	8.500	4.907
3% Alginate	68.433	17.702	10.220
3.5% Alginate	49.433	10.790	6.229

Power of performed test with alpha = 0.050: 0.999

Source of Variation	DF	SS	MS	F	P
Between Treatments	5	5461.689	1092.338	12.282	<0.001
Residual	12	1067.273	88.939		
Total	17	6528.963			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparison	Diff of Means	p	q	P<0.05
DMHP Powder vs. 3.5% Alginate	47.967	6	8.810	Yes
DMHP Powder vs. 3% Alginate	28.967	5	5.320	Yes
DMHP Powder vs. 2.5% Alginate	28.233	4	5.185	Yes
DMHP Powder vs. 2% Alginate	5.667	3	1.041	No
DMHP Powder vs. 1% Alginate	2.933	2	0.539	No
1% Alginate vs. 3.5% Alginate	45.033	5	8.271	Yes
1% Alginate vs. 3% Alginate	26.033	4	4.781	Yes
1% Alginate vs. 2.5% Alginate	25.300	3	4.647	Yes
1% Alginate vs. 2% Alginate	2.733	2	0.502	No
2% Alginate vs. 3.5% Alginate	42.300	4	7.769	Yes
2% Alginate vs. 3% Alginate	23.300	3	4.279	Yes
2% Alginate vs. 2.5% Alginate	22.567	2	4.145	Yes
2.5% Alginate vs. 3.5% Alginate	19.733	3	3.624	No
2.5% Alginate vs. 3% Alginate	0.733	2	0.135	No
3% Alginate vs. 3.5% Alginate	19.000	2	3.490	Yes

Appendix 5: ANOVA and SNK at 2 hour release in phosphate pH=7.4 buffer for formulations 1-5 cured for 30 min and dried at 60^0C

Normality Test: Passed (P = 0.405)

Equal Variance Test: Passed (P = 0.176)

Group	Mean	Std Dev	SEM
DMHP Powder	97.400	1.345	0.777
1% Alginate	96.733	5.645	3.259
2% Alginate	73.800	6.631	3.315
2.5% Alginate	67.500	4.700	2.714
3% Alginate	45.100	1.277	0.737
3.5% Alginate	58.667	2.259	1.304

Power of performed test with alpha = 0.050: 1.000

Source of Variation	DF	SS	MS	F	P
Between Treatments	5	6519.748	1303.950	65.986	<0.001
Residual	13	256.893	19.761		
Total	18	6776.641			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparison	Diff of Means	p	q	P<0.05
DMHP Powder vs. 3% Alginate	52.300	6	20.378	Yes
DMHP Powder vs. 3.5% Alginate	38.733	5	15.092	Yes
DMHP Powder vs. 2.5% Alginate	29.900	4	11.650	Yes
DMHP Powder vs. 2% Alginate	23.600	3	9.830	Yes
DMHP Powder vs. 1% Alginate	0.667	2	0.260	No
1% Alginate vs. 3% Alginate	51.633	5	20.118	Yes
1% Alginate vs. 3.5% Alginate	38.067	4	14.832	Yes
1% Alginate vs. 2.5% Alginate	29.233	3	11.390	Yes
1% Alginate vs. 2% Alginate	22.933	2	9.553	Yes
2% Alginate vs. 3% Alginate	28.700	4	11.955	Yes
2% Alginate vs. 3.5% Alginate	15.133	3	6.304	Yes
2% Alginate vs. 2.5% Alginate	6.300	2	2.624	No
2.5% Alginate vs. 3% Alginate	22.400	3	8.728	Yes
2.5% Alginate vs. 3.5% Alginate	8.833	2	3.442	Yes
3.5% Alginate vs. 3% Alginate	13.567	2	5.286	Yes

Appendix 6: ANOVA and SNK at 2 hour release in phosphate pH=7.4 buffer for formulations 1-5 cured for 45 min and dried at 60^0C

Normality Test: Passed (P = 0.606)

Equal Variance Test: Passed (P = 0.364)

Group	Mean	Std Dev	SEM
DMHP Powder	97.400	1.345	0.777
1% Alginate	91.333	4.271	2.466
2% Alginate	72.100	2.910	1.680
2.5% Alginate	67.867	5.186	2.994
3% Alginate	54.533	6.912	3.991
3.5% Alginate	58.300	2.121	1.500

Power of performed test with alpha = 0.050: 1.000

Source of Variation	DF	SS	MS	F	P
Between Treatments	5	4293.478	858.696	44.792	<0.001
Residual	11	210.880	19.171		
Total	16	4504.358			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparison	Diff of Means	p	q	P<0.05
DMHP Powder vs. 3% Alginate	42.867	6	16.957	Yes
DMHP Powder vs. 3.5% Alginate	39.100	5	13.834	Yes
DMHP Powder vs. 2.5% Alginate	29.533	4	11.683	Yes
DMHP Powder vs. 2% Alginate	25.300	3	10.008	Yes
DMHP Powder vs. 1% Alginate	6.067	2	2.400	No
1% Alginate vs. 3% Alginate	36.800	5	14.558	Yes
1% Alginate vs. 3.5% Alginate	33.033	4	11.688	Yes
1% Alginate vs. 2.5% Alginate	23.467	3	9.283	Yes
1% Alginate vs. 2% Alginate	19.233	2	7.608	Yes
2% Alginate vs. 3% Alginate	17.567	4	6.949	Yes
2% Alginate vs. 3.5% Alginate	13.800	3	4.883	Yes
2% Alginate vs. 2.5% Alginate	4.233	2	1.675	No
2.5% Alginate vs. 3% Alginate	13.333	3	5.274	Yes
2.5% Alginate vs. 3.5% Alginate	9.567	2	3.385	Yes
3.5% Alginate vs. 3% Alginate	3.767	2	1.333	No

Appendix 7: SNK multiple comparison of DMHP release at 2 h. in phosphate pH=7.4 buffer. Legend: % - is the alginate concentration. CT – curing time. DT – drying temperature.

Comparison	Diff of Means	p	q'	P<0.05
1% CT 30 DT 60 vs. 3% CT 30 DT 40	62.900	30	9.759	Yes
1% CT 30 DT 60 vs. 3% CT 45 DT 40	60.933	29	9.453	Yes
1% CT 30 DT 60 vs. 3% CT 30 DT 60	51.633	28	8.011	Yes
1% CT 30 DT 60 vs. 3.5% CT 15 DT 60	47.300	27	7.338	Yes
1% CT 30 DT 60 vs. 3.5% CT 15 DT 40	43.533	26	6.754	Yes
1% CT 30 DT 60 vs. 3% CT 45 DT 60	42.200	25	6.547	Yes
1% CT 30 DT 60 vs. 3.5% CT 45 DT 60	38.433	24	5.333	No
1% CT 30 DT 60 vs. 3.5% CT 30 DT 60	38.067	23	5.906	Yes
1% CT 30 DT 60 vs. 2% CT 30 DT 40	36.208	22	6.005	Yes
1% CT 30 DT 60 vs. 2% CT 45 DT 40	35.467	21	5.502	Yes
1% CT 30 DT 60 vs. 3.5% CT 45 DT 40	29.533	20	4.098	No
1% CT 30 DT 60 vs. 2.5% CT 30 DT 60	29.233	19	4.535	No
1% CT 30 DT 60 vs. 2.5% CT 45 DT 60	28.867	18	4.478	No
1% CT 30 DT 60 vs. 3% CT 15 DT 60	28.300	17	4.391	No
1% CT 30 DT 60 vs. 2.5% CT 15 DT 60	27.567	16	4.277	No
1% CT 30 DT 60 vs. 2% CT 45 DT 60	24.633	15	3.822	No
1% CT 30 DT 60 vs. 2.5% CT 15 DT 40	23.467	14	3.641	No
1% CT 30 DT 60 vs. 2% CT 30 DT 60	22.933	13	3.804	No
1% CT 30 DT 60 vs. 3.5% CT 30 DT 40	22.400	12	3.475	No
1% CT 30 DT 60 vs. 2.5% CT 30 DT 40	21.733	11	3.372	No
1% CT 30 DT 60 vs. 1% CT 30 DT 40	19.967	10	3.098	No
1% CT 30 DT 60 vs. 2.5% CT 45 DT 40	19.667	9	3.051	No
1% CT 30 DT 60 vs. 1% CT 45 DT 40	19.300	8	2.994	No
1% CT 30 DT 60 vs. 3% CT 15 DT 40	15.367	7	2.384	No
1% CT 30 DT 60 vs. 1% CT 45 DT 60	5.400	6	0.838	No
1% CT 30 DT 60 vs. 2% CT 15 DT 60	5.000	5	0.776	No
1% CT 30 DT 60 vs. 1% CT15 DT 40	4.767	4	0.740	No
1% CT 30 DT 60 vs. 2% CT 15 DT 40	3.200	3	0.496	No
1% CT 30 DT 60 vs. 1% CT 15 DT 60	2.267	2	0.352	No
1% CT 15 DT 60 vs. 3% CT 30 DT 40	60.633	29	9.407	Yes
1% CT 15 DT 60 vs. 3% CT 45 DT 40	58.667	28	9.102	Yes
1% CT 15 DT 60 vs. 3% CT 30 DT 60	49.367	27	7.659	Yes
1% CT 15 DT 60 vs. 3.5% CT 15 DT 60	45.033	26	6.987	Yes
1% CT 15 DT 60 vs. 3.5% CT 15 DT 40	41.267	25	6.402	Yes
1% CT 15 DT 60 vs. 3% CT 45 DT 60	39.933	24	6.195	Yes
1% CT 15 DT 60 vs. 3.5% CT 45 DT 60	36.167	23	5.019	No
1% CT 15 DT 60 vs. 3.5% CT 30 DT 60	35.800	22	5.554	Yes
1% CT 15 DT 60 vs. 2% CT 30 DT 40	33.942	21	5.629	Yes
1% CT 15 DT 60 vs. 2% CT 45 DT 40	33.200	20	5.151	No
1% CT 15 DT 60 vs. 3.5% CT 45 DT 40	27.267	19	3.784	No

Comparison	Diff of Means	p	q	P<0.05
1% CT 15 DT 60 vs. 2.5% CT 30 DT 60	26.967	18	4.184	No
1% CT 15 DT 60 vs. 2.5% CT 45 DT 60	26.600	17	4.127	No
1% CT 15 DT 60 vs. 3% CT 15 DT 60	26.033	16	4.039	No
1% CT 15 DT 60 vs. 2.5% CT 15 DT 60	25.300	15	3.925	No
1% CT 15 DT 60 vs. 2% CT 45 DT 60	22.367	14	3.470	No
1% CT 15 DT 60 vs. 2.5% CT 15 DT 40	21.200	13	3.289	No
1% CT 15 DT 60 vs. 2% CT 30 DT 60	20.667	12	3.428	No
1% CT 15 DT 60 vs. 3.5% CT 30 DT 40	20.133	11	3.124	No
1% CT 15 DT 60 vs. 2.5% CT 30 DT 40	19.467	10	3.020	No
1% CT 15 DT 60 vs. 1% CT 30 DT 40	17.700	9	2.746	No
1% CT 15 DT 60 vs. 2.5% CT 45 DT 40	17.400	8	2.700	No
1% CT 15 DT 60 vs. 1% CT 45 DT 40	17.033	7	2.643	No
1% CT 15 DT 60 vs. 3% CT 15 DT 40	13.100	6	2.032	No
1% CT 15 DT 60 vs. 1% CT 45 DT 60	3.133	5	0.486	No
1% CT 15 DT 60 vs. 2% CT 15 DT 60	2.733	4	0.424	No
1% CT 15 DT 60 vs. 1% CT15 DT 40	2.500	3	0.388	No
1% CT 15 DT 60 vs. 2% CT 15 DT 40	0.933	2	0.145	No
2% CT 15 DT 40 vs. 3% CT 30 DT 40	59.700	28	9.262	Yes
2% CT 15 DT 40 vs. 3% CT 45 DT 40	57.733	27	8.957	Yes
2% CT 15 DT 40 vs. 3% CT 30 DT 60	48.433	26	7.514	Yes
2% CT 15 DT 40 vs. 3.5% CT 15 DT 60	44.100	25	6.842	Yes
2% CT 15 DT 40 vs. 3.5% CT 15 DT 40	40.333	24	6.257	Yes
2% CT 15 DT 40 vs. 3% CT 45 DT 60	39.000	23	6.051	Yes
2% CT 15 DT 40 vs. 3.5% CT 45 DT 60	35.233	22	4.889	No
2% CT 15 DT 40 vs. 3.5% CT 30 DT 60	34.867	21	5.409	Yes
2% CT 15 DT 40 vs. 2% CT 30 DT 40	33.008	20	5.475	Yes
2% CT 15 DT 40 vs. 2% CT 45 DT 40	32.267	19	5.006	No
2% CT 15 DT 40 vs. 3.5% CT 45 DT 40	26.333	18	3.654	No
2% CT 15 DT 40 vs. 2.5% CT 30 DT 60	26.033	17	4.039	No
2% CT 15 DT 40 vs. 2.5% CT 45 DT 60	25.667	16	3.982	No
2% CT 15 DT 40 vs. 3% CT 15 DT 60	25.100	15	3.894	No
2% CT 15 DT 40 vs. 2.5% CT 15 DT 60	24.367	14	3.780	No
2% CT 15 DT 40 vs. 2% CT 45 DT 60	21.433	13	3.325	No
2% CT 15 DT 40 vs. 2.5% CT 15 DT 40	20.267	12	3.144	No
2% CT 15 DT 40 vs. 2% CT 30 DT 60	19.733	11	3.273	No
2% CT 15 DT 40 vs. 3.5% CT 30 DT 40	19.200	10	2.979	No
2% CT 15 DT 40 vs. 2.5% CT 30 DT 40	18.533	9	2.875	No
2% CT 15 DT 40 vs. 1% CT 30 DT 40	16.767	8	2.601	No
2% CT 15 DT 40 vs. 2.5% CT 45 DT 40	16.467	7	2.555	No
2% CT 15 DT 40 vs. 1% CT 45 DT 40	16.100	6	2.498	No
2% CT 15 DT 40 vs. 3% CT 15 DT 40	12.167	5	1.888	No
2% CT 15 DT 40 vs. 1% CT 45 DT 60	2.200	4	0.341	No
2% CT 15 DT 40 vs. 2% CT 15 DT 60	1.800	3	0.279	No
2% CT 15 DT 40 vs. 1% CT15 DT 40	1.567	2	0.243	No

Comparison	Diff of Means	p	q	P<0.05
1% CT15 DT 40 vs. 3% CT 30 DT 40	58.133	27	9.019	Yes
1% CT15 DT 40 vs. 3% CT 45 DT 40	56.167	26	8.714	Yes
1% CT15 DT 40 vs. 3% CT 30 DT 60	46.867	25	7.271	Yes
1% CT15 DT 40 vs. 3.5% CT 15 DT 60	42.533	24	6.599	Yes
1% CT15 DT 40 vs. 3.5% CT 15 DT 40	38.767	23	6.014	Yes
1% CT15 DT 40 vs. 3% CT 45 DT 60	37.433	22	5.808	Yes
1% CT15 DT 40 vs. 3.5% CT 45 DT 60	33.667	21	4.672	No
1% CT15 DT 40 vs. 3.5% CT 30 DT 60	33.300	20	5.166	No
1% CT15 DT 40 vs. 2% CT 30 DT 40	31.442	19	5.215	Yes
1% CT15 DT 40 vs. 2% CT 45 DT 40	30.700	18	4.763	No
1% CT15 DT 40 vs. 3.5% CT 45 DT 40	24.767	17	3.437	No
1% CT15 DT 40 vs. 2.5% CT 30 DT 60	24.467	16	3.796	No
1% CT15 DT 40 vs. 2.5% CT 45 DT 60	24.100	15	3.739	No
1% CT15 DT 40 vs. 3% CT 15 DT 60	23.533	14	3.651	No
1% CT15 DT 40 vs. 2.5% CT 15 DT 60	22.800	13	3.537	No
1% CT15 DT 40 vs. 2% CT 45 DT 60	19.867	12	3.082	No
1% CT15 DT 40 vs. 2.5% CT 15 DT 40	18.700	11	2.901	No
1% CT15 DT 40 vs. 2% CT 30 DT 60	18.167	10	3.013	No
1% CT15 DT 40 vs. 3.5% CT 30 DT 40	17.633	9	2.736	No
1% CT15 DT 40 vs. 2.5% CT 30 DT 40	16.967	8	2.632	No
1% CT15 DT 40 vs. 1% CT 30 DT 40	15.200	7	2.358	No
1% CT15 DT 40 vs. 2.5% CT 45 DT 40	14.900	6	2.312	No
1% CT15 DT 40 vs. 1% CT 45 DT 40	14.533	5	2.255	No
1% CT15 DT 40 vs. 3% CT 15 DT 40	10.600	4	1.645	No
1% CT15 DT 40 vs. 1% CT 45 DT 60	0.633	3	0.098	No
1% CT15 DT 40 vs. 2% CT 15 DT 60	0.233	2	0.036	No
2% CT 15 DT 60 vs. 3% CT 30 DT 40	57.900	26	8.983	Yes
2% CT 15 DT 60 vs. 3% CT 45 DT 40	55.933	25	8.678	Yes
2% CT 15 DT 60 vs. 3% CT 30 DT 60	46.633	24	7.235	Yes
2% CT 15 DT 60 vs. 3.5% CT 15 DT 60	42.300	23	6.563	Yes
2% CT 15 DT 60 vs. 3.5% CT 15 DT 40	38.533	22	5.978	Yes
2% CT 15 DT 60 vs. 3% CT 45 DT 60	37.200	21	5.771	Yes
2% CT 15 DT 60 vs. 3.5% CT 45 DT 60	33.433	20	4.639	No
2% CT 15 DT 60 vs. 3.5% CT 30 DT 60	33.067	19	5.130	No
2% CT 15 DT 60 vs. 2% CT 30 DT 40	31.208	18	5.176	Yes
2% CT 15 DT 60 vs. 2% CT 45 DT 40	30.467	17	4.727	No
2% CT 15 DT 60 vs. 3.5% CT 45 DT 40	24.533	16	3.404	No
2% CT 15 DT 60 vs. 2.5% CT 30 DT 60	24.233	15	3.760	No
2% CT 15 DT 60 vs. 2.5% CT 45 DT 60	23.867	14	3.703	No
2% CT 15 DT 60 vs. 3% CT 15 DT 60	23.300	13	3.615	No
2% CT 15 DT 60 vs. 2.5% CT 15 DT 60	22.567	12	3.501	No
2% CT 15 DT 60 vs. 2% CT 45 DT 60	19.633	11	3.046	No
2% CT 15 DT 60 vs. 2.5% CT 15 DT 40	18.467	10	2.865	No
2% CT 15 DT 60 vs. 2% CT 30 DT 60	17.933	9	2.974	No

Comparison	Diff of Means	p	q	P<0.05
2% CT 15 DT 60 vs. 3.5% CT 30 DT 40	17.400	8	2.700	No
2% CT 15 DT 60 vs. 2.5% CT 30 DT 40	16.733	7	2.596	No
2% CT 15 DT 60 vs. 1% CT 30 DT 40	14.967	6	2.322	No
2% CT 15 DT 60 vs. 2.5% CT 45 DT 40	14.667	5	2.275	No
2% CT 15 DT 60 vs. 1% CT 45 DT 40	14.300	4	2.219	No
2% CT 15 DT 60 vs. 3% CT 15 DT 40	10.367	3	1.608	No
2% CT 15 DT 60 vs. 1% CT 45 DT 60	0.400	2	0.0621	No
1% CT 45 DT 60 vs. 3% CT 30 DT 40	57.500	25	8.921	Yes
1% CT 45 DT 60 vs. 3% CT 45 DT 40	55.533	24	8.616	Yes
1% CT 45 DT 60 vs. 3% CT 30 DT 60	46.233	23	7.173	Yes
1% CT 45 DT 60 vs. 3.5% CT 15 DT 60	41.900	22	6.501	Yes
1% CT 45 DT 60 vs. 3.5% CT 15 DT 40	38.133	21	5.916	Yes
1% CT 45 DT 60 vs. 3% CT 45 DT 60	36.800	20	5.709	Yes
1% CT 45 DT 60 vs. 3.5% CT 45 DT 60	33.033	19	4.584	No
1% CT 45 DT 60 vs. 3.5% CT 30 DT 60	32.667	18	5.068	No
1% CT 45 DT 60 vs. 2% CT 30 DT 40	30.808	17	5.110	Yes
1% CT 45 DT 60 vs. 2% CT 45 DT 40	30.067	16	4.665	No
1% CT 45 DT 60 vs. 3.5% CT 45 DT 40	24.133	15	3.349	No
1% CT 45 DT 60 vs. 2.5% CT 30 DT 60	23.833	14	3.698	No
1% CT 45 DT 60 vs. 2.5% CT 45 DT 60	23.467	13	3.641	No
1% CT 45 DT 60 vs. 3% CT 15 DT 60	22.900	12	3.553	No
1% CT 45 DT 60 vs. 2.5% CT 15 DT 60	22.167	11	3.439	No
1% CT 45 DT 60 vs. 2% CT 45 DT 60	19.233	10	2.984	No
1% CT 45 DT 60 vs. 2.5% CT 15 DT 40	18.067	9	2.803	No
1% CT 45 DT 60 vs. 2% CT 30 DT 60	17.533	8	2.908	No
1% CT 45 DT 60 vs. 3.5% CT 30 DT 40	17.000	7	2.637	No
1% CT 45 DT 60 vs. 2.5% CT 30 DT 40	16.333	6	2.534	No
1% CT 45 DT 60 vs. 1% CT 30 DT 40	14.567	5	2.260	No
1% CT 45 DT 60 vs. 2.5% CT 45 DT 40	14.267	4	2.213	No
1% CT 45 DT 60 vs. 1% CT 45 DT 40	13.900	3	2.157	No
1% CT 45 DT 60 vs. 3% CT 15 DT 40	9.967	2	1.546	No
3% CT 15 DT 40 vs. 3% CT 30 DT 40	47.533	24	7.375	Yes
3% CT 15 DT 40 vs. 3% CT 45 DT 40	45.567	23	7.069	Yes
3% CT 15 DT 40 vs. 3% CT 30 DT 60	36.267	22	5.627	Yes
3% CT 15 DT 40 vs. 3.5% CT 15 DT 60	31.933	21	4.954	No
3% CT 15 DT 40 vs. 3.5% CT 15 DT 40	28.167	20	4.370	No
3% CT 15 DT 40 vs. 3% CT 45 DT 60	26.833	19	4.163	No
3% CT 15 DT 40 vs. 3.5% CT 45 DT 60	23.067	18	3.201	No
3% CT 15 DT 40 vs. 3.5% CT 30 DT 60	22.700	17	3.522	No
3% CT 15 DT 40 vs. 2% CT 30 DT 40	20.842	16	3.457	No
3% CT 15 DT 40 vs. 2% CT 45 DT 40	20.100	15	3.118	No
3% CT 15 DT 40 vs. 3.5% CT 45 DT 40	14.167	14	1.966	No
3% CT 15 DT 40 vs. 2.5% CT 30 DT 60	13.867	13	2.151	No
3% CT 15 DT 40 vs. 2.5% CT 45 DT 60	13.500	12	2.094	No

Comparison	Diff of Means	p	q	P<0.05
3% CT 15 DT 40 vs. 3% CT 15 DT 60	12.933	11	2.007	No
3% CT 15 DT 40 vs. 2.5% CT 15 DT 60	12.200	10	1.893	No
3% CT 15 DT 40 vs. 2% CT 45 DT 60	9.267	9	1.438	No
3% CT 15 DT 40 vs. 2.5% CT 15 DT 40	8.100	8	1.257	No
3% CT 15 DT 40 vs. 2% CT 30 DT 60	7.567	7	1.255	No
3% CT 15 DT 40 vs. 3.5% CT 30 DT 40	7.033	6	1.091	No
3% CT 15 DT 40 vs. 2.5% CT 30 DT 40	6.367	5	0.988	No
3% CT 15 DT 40 vs. 1% CT 30 DT 40	4.600	4	0.714	No
3% CT 15 DT 40 vs. 2.5% CT 45 DT 40	4.300	3	0.667	No
3% CT 15 DT 40 vs. 1% CT 45 DT 40	3.933	2	0.610	No
1% CT 45 DT 40 vs. 3% CT 30 DT 40	43.600	23	6.764	Yes
1% CT 45 DT 40 vs. 3% CT 45 DT 40	41.633	22	6.459	Yes
1% CT 45 DT 40 vs. 3% CT 30 DT 60	32.333	21	5.016	No
1% CT 45 DT 40 vs. 3.5% CT 15 DT 60	28.000	20	4.344	No
1% CT 45 DT 40 vs. 3.5% CT 15 DT 40	24.233	19	3.760	No
1% CT 45 DT 40 vs. 3% CT 45 DT 60	22.900	18	3.553	No
1% CT 45 DT 40 vs. 3.5% CT 45 DT 60	19.133	17	2.655	No
1% CT 45 DT 40 vs. 3.5% CT 30 DT 60	18.767	16	2.912	No
1% CT 45 DT 40 vs. 2% CT 30 DT 40	16.908	15	2.804	No
1% CT 45 DT 40 vs. 2% CT 45 DT 40	16.167	14	2.508	No
1% CT 45 DT 40 vs. 3.5% CT 45 DT 40	10.233	13	1.420	No
1% CT 45 DT 40 vs. 2.5% CT 30 DT 60	9.933	12	1.541	No
1% CT 45 DT 40 vs. 2.5% CT 45 DT 60	9.567	11	1.484	No
1% CT 45 DT 40 vs. 3% CT 15 DT 60	9.000	10	1.396	No
1% CT 45 DT 40 vs. 2.5% CT 15 DT 60	8.267	9	1.283	No
1% CT 45 DT 40 vs. 2% CT 45 DT 60	5.333	8	0.827	No
1% CT 45 DT 40 vs. 2.5% CT 15 DT 40	4.167	7	0.646	No
1% CT 45 DT 40 vs. 2% CT 30 DT 60	3.633	6	0.603	No
1% CT 45 DT 40 vs. 3.5% CT 30 DT 40	3.100	5	0.481	No
1% CT 45 DT 40 vs. 2.5% CT 30 DT 40	2.433	4	0.378	No
1% CT 45 DT 40 vs. 1% CT 30 DT 40	0.667	3	0.103	No
1% CT 45 DT 40 vs. 2.5% CT 45 DT 40	0.367	2	0.056	No
2.5% CT 45 DT 4 vs. 3% CT 30 DT 40	43.233	22	6.707	Yes
2.5% CT 45 DT 4 vs. 3% CT 45 DT 40	41.267	21	6.402	Yes
2.5% CT 45 DT 4 vs. 3% CT 30 DT 60	31.967	20	4.959	No
2.5% CT 45 DT 4 vs. 3.5% CT 15 DT 60	27.633	19	4.287	No
2.5% CT 45 DT 4 vs. 3.5% CT 15 DT 40	23.867	18	3.703	No
2.5% CT 45 DT 4 vs. 3% CT 45 DT 60	22.533	17	3.496	No
2.5% CT 45 DT 4 vs. 3.5% CT 45 DT 60	18.767	16	2.604	No
2.5% CT 45 DT 4 vs. 3.5% CT 30 DT 60	18.400	15	2.855	No
2.5% CT 45 DT 4 vs. 2% CT 30 DT 40	16.542	14	2.744	No
2.5% CT 45 DT 4 vs. 2% CT 45 DT 40	15.800	13	2.451	No
2.5% CT 45 DT 4 vs. 3.5% CT 45 DT 40	9.867	12	1.369	No
2.5% CT 45 DT 4 vs. 2.5% CT 30 DT 60	9.567	11	1.484	No

Comparison	Diff of Means	p	q	P<0.05
2.5% CT 45 DT 4 vs. 2.5% CT 45 DT 60	9.200	10	1.427	No
2.5% CT 45 DT 4 vs. 3% CT 15 DT 60	8.633	9	1.339	No
2.5% CT 45 DT 4 vs. 2.5% CT 15 DT 60	7.900	8	1.226	No
2.5% CT 45 DT 4 vs. 2% CT 45 DT 60	4.967	7	0.771	No
2.5% CT 45 DT 4 vs. 2.5% CT 15 DT 40	3.800	6	0.590	No
2.5% CT 45 DT 4 vs. 2% CT 30 DT 60	3.267	5	0.542	No
2.5% CT 45 DT 4 vs. 3.5% CT 30 DT 40	2.733	4	0.424	No
2.5% CT 45 DT 4 vs. 2.5% CT 30 DT 40	2.067	3	0.321	No
2.5% CT 45 DT 4 vs. 1% CT 30 DT 40	0.300	2	0.0465	No
1% CT 30 DT 40 vs. 3% CT 30 DT 40	42.933	21	6.661	Yes
1% CT 30 DT 40 vs. 3% CT 45 DT 40	40.967	20	6.356	Yes
1% CT 30 DT 40 vs. 3% CT 30 DT 60	31.667	19	4.913	No
1% CT 30 DT 40 vs. 3.5% CT 15 DT 60	27.333	18	4.241	No
1% CT 30 DT 40 vs. 3.5% CT 15 DT 40	23.567	17	3.656	No
1% CT 30 DT 40 vs. 3% CT 45 DT 60	22.233	16	3.449	No
1% CT 30 DT 40 vs. 3.5% CT 45 DT 60	18.467	15	2.563	No
1% CT 30 DT 40 vs. 3.5% CT 30 DT 60	18.100	14	2.808	No
1% CT 30 DT 40 vs. 2% CT 30 DT 40	16.242	13	2.694	No
1% CT 30 DT 40 vs. 2% CT 45 DT 40	15.500	12	2.405	No
1% CT 30 DT 40 vs. 3.5% CT 45 DT 40	9.567	11	1.328	No
1% CT 30 DT 40 vs. 2.5% CT 30 DT 60	9.267	10	1.438	No
1% CT 30 DT 40 vs. 2.5% CT 45 DT 60	8.900	9	1.381	No
1% CT 30 DT 40 vs. 3% CT 15 DT 60	8.333	8	1.293	No
1% CT 30 DT 40 vs. 2.5% CT 15 DT 60	7.600	7	1.179	No
1% CT 30 DT 40 vs. 2% CT 45 DT 60	4.667	6	0.724	No
1% CT 30 DT 40 vs. 2.5% CT 15 DT 40	3.500	5	0.543	No
1% CT 30 DT 40 vs. 2% CT 30 DT 60	2.967	4	0.492	No
1% CT 30 DT 40 vs. 3.5% CT 30 DT 40	2.433	3	0.378	No
1% CT 30 DT 40 vs. 2.5% CT 30 DT 40	1.767	2	0.274	No
2.5% CT 30 DT 4 vs. 3% CT 30 DT 40	41.167	20	6.387	Yes
2.5% CT 30 DT 4 vs. 3% CT 45 DT 40	39.200	19	6.082	Yes
2.5% CT 30 DT 4 vs. 3% CT 30 DT 60	29.900	18	4.639	No
2.5% CT 30 DT 4 vs. 3.5% CT 15 DT 60	25.567	17	3.967	No
2.5% CT 30 DT 4 vs. 3.5% CT 15 DT 40	21.800	16	3.382	No
2.5% CT 30 DT 4 vs. 3% CT 45 DT 60	20.467	15	3.175	No
2.5% CT 30 DT 4 vs. 3.5% CT 45 DT 60	16.700	14	2.317	No
2.5% CT 30 DT 4 vs. 3.5% CT 30 DT 60	16.333	13	2.534	No
2.5% CT 30 DT 4 vs. 2% CT 30 DT 40	14.475	12	2.401	No
2.5% CT 30 DT 4 vs. 2% CT 45 DT 40	13.733	11	2.131	No
2.5% CT 30 DT 4 vs. 3.5% CT 45 DT 40	7.800	10	1.082	No
2.5% CT 30 DT 4 vs. 2.5% CT 30 DT 60	7.500	9	1.164	No
2.5% CT 30 DT 4 vs. 2.5% CT 45 DT 60	7.133	8	1.107	No
2.5% CT 30 DT 4 vs. 3% CT 15 DT 60	6.567	7	1.019	No
2.5% CT 30 DT 4 vs. 2.5% CT 15 DT 60	5.833	6	0.905	No

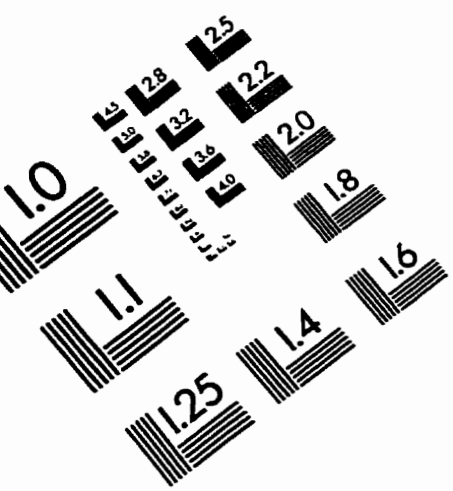
Comparison	Diff of Means	p	q	P<0.05
2.5% CT 30 DT 4 vs. 2% CT 45 DT 60	2.900	5	0.450	No
2.5% CT 30 DT 4 vs. 2.5% CT 15 DT 40	1.733	4	0.269	No
2.5% CT 30 DT 4 vs. 2% CT 30 DT 60	1.200	3	0.199	No
2.5% CT 30 DT 4 vs. 3.5% CT 30 DT 40	0.667	2	0.103	No
3.5% CT 30 DT 4 vs. 3% CT 30 DT 40	40.500	19	6.283	Yes
3.5% CT 30 DT 4 vs. 3% CT 45 DT 40	38.533	18	5.978	Yes
3.5% CT 30 DT 4 vs. 3% CT 30 DT 60	29.233	17	4.535	No
3.5% CT 30 DT 4 vs. 3.5% CT 15 DT 60	24.900	16	3.863	No
3.5% CT 30 DT 4 vs. 3.5% CT 15 DT 40	21.133	15	3.279	No
3.5% CT 30 DT 4 vs. 3% CT 45 DT 60	19.800	14	3.072	No
3.5% CT 30 DT 4 vs. 3.5% CT 45 DT 60	16.033	13	2.225	No
3.5% CT 30 DT 4 vs. 3.5% CT 30 DT 60	15.667	12	2.431	No
3.5% CT 30 DT 4 vs. 2% CT 30 DT 40	13.808	11	2.290	No
3.5% CT 30 DT 4 vs. 2% CT 45 DT 40	13.067	10	2.027	No
3.5% CT 30 DT 4 vs. 3.5% CT 45 DT 40	7.133	9	0.990	No
3.5% CT 30 DT 4 vs. 2.5% CT 30 DT 60	6.833	8	1.060	No
3.5% CT 30 DT 4 vs. 2.5% CT 45 DT 60	6.467	7	1.003	No
3.5% CT 30 DT 4 vs. 3% CT 15 DT 60	5.900	6	0.915	No
3.5% CT 30 DT 4 vs. 2.5% CT 15 DT 60	5.167	5	0.802	No
3.5% CT 30 DT 4 vs. 2% CT 45 DT 60	2.233	4	0.346	No
3.5% CT 30 DT 4 vs. 2.5% CT 15 DT 40	1.067	3	0.165	No
3.5% CT 30 DT 4 vs. 2% CT 30 DT 60	0.533	2	0.0885	No
2% CT 30 DT 60 vs. 3% CT 30 DT 40	39.967	18	6.629	Yes
2% CT 30 DT 60 vs. 3% CT 45 DT 40	38.000	17	6.303	Yes
2% CT 30 DT 60 vs. 3% CT 30 DT 60	28.700	16	4.760	No
2% CT 30 DT 60 vs. 3.5% CT 15 DT 60	24.367	15	4.041	No
2% CT 30 DT 60 vs. 3.5% CT 15 DT 40	20.600	14	3.417	No
2% CT 30 DT 60 vs. 3% CT 45 DT 60	19.267	13	3.195	No
2% CT 30 DT 60 vs. 3.5% CT 45 DT 60	15.500	12	2.267	No
2% CT 30 DT 60 vs. 3.5% CT 30 DT 60	15.133	11	2.510	No
2% CT 30 DT 60 vs. 2% CT 30 DT 40	13.275	10	2.378	No
2% CT 30 DT 60 vs. 2% CT 45 DT 40	12.533	9	2.079	No
2% CT 30 DT 60 vs. 3.5% CT 45 DT 40	6.600	8	0.965	No
2% CT 30 DT 60 vs. 2.5% CT 30 DT 60	6.300	7	1.045	No
2% CT 30 DT 60 vs. 2.5% CT 45 DT 60	5.933	6	0.984	No
2% CT 30 DT 60 vs. 3% CT 15 DT 60	5.367	5	0.890	No
2% CT 30 DT 60 vs. 2.5% CT 15 DT 60	4.633	4	0.768	No
2% CT 30 DT 60 vs. 2% CT 45 DT 60	1.700	3	0.282	No
2% CT 30 DT 60 vs. 2.5% CT 15 DT 40	0.533	2	0.0885	No
2.5% CT 15 DT 4 vs. 3% CT 30 DT 40	39.433	17	6.118	Yes
2.5% CT 15 DT 4 vs. 3% CT 45 DT 40	37.467	16	5.813	Yes
2.5% CT 15 DT 4 vs. 3% CT 30 DT 60	28.167	15	4.370	No
2.5% CT 15 DT 4 vs. 3.5% CT 15 DT 60	23.833	14	3.698	No
2.5% CT 15 DT 4 vs. 3.5% CT 15 DT 40	20.067	13	3.113	No

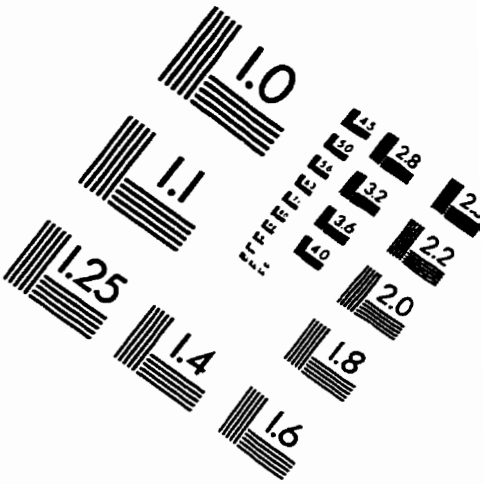
Comparison	Diff of Means	p	q	P<0.05
2.5% CT 15 DT 4 vs. 3% CT 45 DT 60	18.733	12	2.906	No
2.5% CT 15 DT 4 vs. 3.5% CT 45 DT 60	14.967	11	2.077	No
2.5% CT 15 DT 4 vs. 3.5% CT 30 DT 60	14.600	10	2.265	No
2.5% CT 15 DT 4 vs. 2% CT 30 DT 40	12.742	9	2.113	No
2.5% CT 15 DT 4 vs. 2% CT 45 DT 40	12.000	8	1.862	No
2.5% CT 15 DT 4 vs. 3.5% CT 45 DT 40	6.067	7	0.842	No
2.5% CT 15 DT 4 vs. 2.5% CT 30 DT 60	5.767	6	0.895	No
2.5% CT 15 DT 4 vs. 2.5% CT 45 DT 60	5.400	5	0.838	No
2.5% CT 15 DT 4 vs. 3% CT 15 DT 60	4.833	4	0.750	No
2.5% CT 15 DT 4 vs. 2.5% CT 15 DT 60	4.100	3	0.636	No
2.5% CT 15 DT 4 vs. 2% CT 45 DT 60	1.167	2	0.181	No
2% CT 45 DT 60 vs. 3% CT 30 DT 40	38.267	16	5.937	Yes
2% CT 45 DT 60 vs. 3% CT 45 DT 40	36.300	15	5.632	Yes
2% CT 45 DT 60 vs. 3% CT 30 DT 60	27.000	14	4.189	No
2% CT 45 DT 60 vs. 3.5% CT 15 DT 60	22.667	13	3.517	No
2% CT 45 DT 60 vs. 3.5% CT 15 DT 40	18.900	12	2.932	No
2% CT 45 DT 60 vs. 3% CT 45 DT 60	17.567	11	2.725	No
2% CT 45 DT 60 vs. 3.5% CT 45 DT 60	13.800	10	1.915	No
2% CT 45 DT 60 vs. 3.5% CT 30 DT 60	13.433	9	2.084	No
2% CT 45 DT 60 vs. 2% CT 30 DT 40	11.575	8	1.920	No
2% CT 45 DT 60 vs. 2% CT 45 DT 40	10.833	7	1.681	No
2% CT 45 DT 60 vs. 3.5% CT 45 DT 40	4.900	6	0.680	No
2% CT 45 DT 60 vs. 2.5% CT 30 DT 60	4.600	5	0.714	No
2% CT 45 DT 60 vs. 2.5% CT 45 DT 60	4.233	4	0.657	No
2% CT 45 DT 60 vs. 3% CT 15 DT 60	3.667	3	0.569	No
2% CT 45 DT 60 vs. 2.5% CT 15 DT 60	2.933	2	0.455	No
2.5% CT 15 DT 6 vs. 3% CT 30 DT 40	35.333	15	5.482	Yes
2.5% CT 15 DT 6 vs. 3% CT 45 DT 40	33.367	14	5.177	Yes
2.5% CT 15 DT 6 vs. 3% CT 30 DT 60	24.067	13	3.734	No
2.5% CT 15 DT 6 vs. 3.5% CT 15 DT 60	19.733	12	3.062	No
2.5% CT 15 DT 6 vs. 3.5% CT 15 DT 40	15.967	11	2.477	No
2.5% CT 15 DT 6 vs. 3% CT 45 DT 60	14.633	10	2.270	No
2.5% CT 15 DT 6 vs. 3.5% CT 45 DT 60	10.867	9	1.508	No
2.5% CT 15 DT 6 vs. 3.5% CT 30 DT 60	10.500	8	1.629	No
2.5% CT 15 DT 6 vs. 2% CT 30 DT 40	8.642	7	1.433	No
2.5% CT 15 DT 6 vs. 2% CT 45 DT 40	7.900	6	1.226	No
2.5% CT 15 DT 6 vs. 3.5% CT 45 DT 40	1.967	5	0.273	No
2.5% CT 15 DT 6 vs. 2.5% CT 30 DT 60	1.667	4	0.259	No
2.5% CT 15 DT 6 vs. 2.5% CT 45 DT 60	1.300	3	0.202	No
2.5% CT 15 DT 6 vs. 3% CT 15 DT 60	0.733	2	0.114	No
3% CT 15 DT 60 vs. 3% CT 30 DT 40	34.600	14	5.368	Yes
3% CT 15 DT 60 vs. 3% CT 45 DT 40	32.633	13	5.063	Yes
3% CT 15 DT 60 vs. 3% CT 30 DT 60	23.333	12	3.620	No
3% CT 15 DT 60 vs. 3.5% CT 15 DT 60	19.000	11	2.948	No

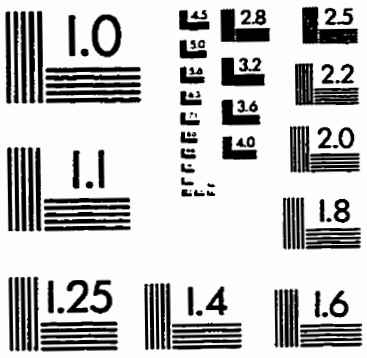
Comparison	Diff of Means	p	q	P<0.05
3% CT 15 DT 60 vs. 3.5% CT 15 DT 40	15.233	10	2.363	No
3% CT 15 DT 60 vs. 3% CT 45 DT 60	13.900	9	2.157	No
3% CT 15 DT 60 vs. 3.5% CT 45 DT 60	10.133	8	1.406	No
3% CT 15 DT 60 vs. 3.5% CT 30 DT 60	9.767	7	1.515	No
3% CT 15 DT 60 vs. 2% CT 30 DT 40	7.908	6	1.312	No
3% CT 15 DT 60 vs. 2% CT 45 DT 40	7.167	5	1.112	No
3% CT 15 DT 60 vs. 3.5% CT 45 DT 40	1.233	4	0.171	No
3% CT 15 DT 60 vs. 2.5% CT 30 DT 60	0.933	3	0.145	No
3% CT 15 DT 60 vs. 2.5% CT 45 DT 60	0.567	2	0.0879	No
2.5% CT 45 DT 6 vs. 3% CT 30 DT 40	34.033	13	5.280	Yes
2.5% CT 45 DT 6 vs. 3% CT 45 DT 40	32.067	12	4.975	Yes
2.5% CT 45 DT 6 vs. 3% CT 30 DT 60	22.767	11	3.532	No
2.5% CT 45 DT 6 vs. 3.5% CT 15 DT 60	18.433	10	2.860	No
2.5% CT 45 DT 6 vs. 3.5% CT 15 DT 40	14.667	9	2.275	No
2.5% CT 45 DT 6 vs. 3% CT 45 DT 60	13.333	8	2.069	No
2.5% CT 45 DT 6 vs. 3.5% CT 45 DT 60	9.567	7	1.328	No
2.5% CT 45 DT 6 vs. 3.5% CT 30 DT 60	9.200	6	1.427	No
2.5% CT 45 DT 6 vs. 2% CT 30 DT 40	7.342	5	1.218	No
2.5% CT 45 DT 6 vs. 2% CT 45 DT 40	6.600	4	1.024	No
2.5% CT 45 DT 6 vs. 3.5% CT 45 DT 40	0.667	3	0.0925	No
2.5% CT 45 DT 6 vs. 2.5% CT 30 DT 60	0.367	2	0.0569	No
2.5% CT 30 DT 6 vs. 3% CT 30 DT 40	33.667	12	5.223	Yes
2.5% CT 30 DT 6 vs. 3% CT 45 DT 40	31.700	11	4.918	Yes
2.5% CT 30 DT 6 vs. 3% CT 30 DT 60	22.400	10	3.475	No
2.5% CT 30 DT 6 vs. 3.5% CT 15 DT 60	18.067	9	2.803	No
2.5% CT 30 DT 6 vs. 3.5% CT 15 DT 40	14.300	8	2.219	No
2.5% CT 30 DT 6 vs. 3% CT 45 DT 60	12.967	7	2.012	No
2.5% CT 30 DT 6 vs. 3.5% CT 45 DT 60	9.200	6	1.277	No
2.5% CT 30 DT 6 vs. 3.5% CT 30 DT 60	8.833	5	1.370	No
2.5% CT 30 DT 6 vs. 2% CT 30 DT 40	6.975	4	1.157	No
2.5% CT 30 DT 6 vs. 2% CT 45 DT 40	6.233	3	0.967	No
2.5% CT 30 DT 6 vs. 3.5% CT 45 DT 40	0.300	2	0.0416	No
3.5% CT 45 DT 4 vs. 3% CT 30 DT 40	33.367	11	4.630	No
3.5% CT 45 DT 4 vs. 3% CT 45 DT 40	31.400	10	4.357	No
3.5% CT 45 DT 4 vs. 3% CT 30 DT 60	22.100	9	3.067	No
3.5% CT 45 DT 4 vs. 3.5% CT 15 DT 60	17.767	8	2.465	No
3.5% CT 45 DT 4 vs. 3.5% CT 15 DT 40	14.000	7	1.943	No
3.5% CT 45 DT 4 vs. 3% CT 45 DT 60	12.667	6	1.758	No
3.5% CT 45 DT 4 vs. 3.5% CT 45 DT 60	8.900	5	1.127	No
3.5% CT 45 DT 4 vs. 3.5% CT 30 DT 60	8.533	4	1.184	No
3.5% CT 45 DT 4 vs. 2% CT 30 DT 40	6.675	3	0.976	No
3.5% CT 45 DT 4 vs. 2% CT 45 DT 40	5.933	2	0.823	No
2% CT 45 DT 40 vs. 3% CT 30 DT 40	27.433	10	4.256	No
2% CT 45 DT 40 vs. 3% CT 45 DT 40	25.467	9	3.951	No

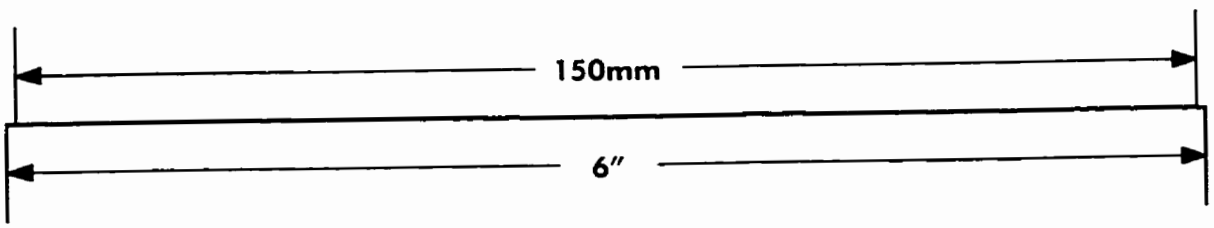
Comparison	Diff of Means	p	q	P<0.05
2% CT 45 DT 40 vs. 3% CT 30 DT 60	16.167	8	2.508	No
2% CT 45 DT 40 vs. 3.5% CT 15 DT 60	11.833	7	1.836	No
2% CT 45 DT 40 vs. 3.5% CT 15 DT 40	8.067	6	1.251	No
2% CT 45 DT 40 vs. 3% CT 45 DT 60	6.733	5	1.045	No
2% CT 45 DT 40 vs. 3.5% CT 45 DT 60	2.967	4	0.412	No
2% CT 45 DT 40 vs. 3.5% CT 30 DT 60	2.600	3	0.403	No
2% CT 45 DT 40 vs. 2% CT 30 DT 40	0.742	2	0.123	No
2% CT 30 DT 40 vs. 3% CT 30 DT 40	26.692	9	4.427	No
2% CT 30 DT 40 vs. 3% CT 45 DT 40	24.725	8	4.101	No
2% CT 30 DT 40 vs. 3% CT 30 DT 60	15.425	7	2.558	No
2% CT 30 DT 40 vs. 3.5% CT 15 DT 60	11.092	6	1.840	No
2% CT 30 DT 40 vs. 3.5% CT 15 DT 40	7.325	5	1.215	No
2% CT 30 DT 40 vs. 3% CT 45 DT 60	5.992	4	0.994	No
2% CT 30 DT 40 vs. 3.5% CT 45 DT 60	2.225	3	0.325	No
2% CT 30 DT 40 vs. 3.5% CT 30 DT 60	1.858	2	0.308	No
3.5% CT 30 DT 6 vs. 3% CT 30 DT 40	24.833	8	3.853	No
3.5% CT 30 DT 6 vs. 3% CT 45 DT 40	22.867	7	3.548	No
3.5% CT 30 DT 6 vs. 3% CT 30 DT 60	13.567	6	2.105	No
3.5% CT 30 DT 6 vs. 3.5% CT 15 DT 60	9.233	5	1.432	No
3.5% CT 30 DT 6 vs. 3.5% CT 15 DT 40	5.467	4	0.848	No
3.5% CT 30 DT 6 vs. 3% CT 45 DT 60	4.133	3	0.641	No
3.5% CT 30 DT 6 vs. 3.5% CT 45 DT 60	0.367	2	0.0509	No
3.5% CT 45 DT 6 vs. 3% CT 30 DT 40	24.467	7	3.395	No
3.5% CT 45 DT 6 vs. 3% CT 45 DT 40	22.500	6	3.122	No
3.5% CT 45 DT 6 vs. 3% CT 30 DT 60	13.200	5	1.832	No
3.5% CT 45 DT 6 vs. 3.5% CT 15 DT 60	8.867	4	1.230	No
3.5% CT 45 DT 6 vs. 3.5% CT 15 DT 40	5.100	3	0.708	No
3.5% CT 45 DT 6 vs. 3% CT 45 DT 60	3.767	2	0.523	No
3% CT 45 DT 60 vs. 3% CT 30 DT 40	20.700	6	3.211	No
3% CT 45 DT 60 vs. 3% CT 45 DT 40	18.733	5	2.906	No
3% CT 45 DT 60 vs. 3% CT 30 DT 60	9.433	4	1.464	No
3% CT 45 DT 60 vs. 3.5% CT 15 DT 60	5.100	3	0.791	No
3% CT 45 DT 60 vs. 3.5% CT 15 DT 40	1.333	2	0.207	No
3.5% CT 15 DT 4 vs. 3% CT 30 DT 40	19.367	5	3.005	No
3.5% CT 15 DT 4 vs. 3% CT 45 DT 40	17.400	4	2.700	No
3.5% CT 15 DT 4 vs. 3% CT 30 DT 60	8.100	3	1.257	No
3.5% CT 15 DT 4 vs. 3.5% CT 15 DT 60	3.767	2	0.584	No
3.5% CT 15 DT 6 vs. 3% CT 30 DT 40	15.600	4	2.420	No
3.5% CT 15 DT 6 vs. 3% CT 45 DT 40	13.633	3	2.115	No
3.5% CT 15 DT 6 vs. 3% CT 30 DT 60	4.333	2	0.672	No
3% CT 30 DT 60 vs. 3% CT 30 DT 40	11.267	3	1.748	No
3% CT 30 DT 60 vs. 3% CT 45 DT 40	9.300	2	1.443	No
3% CT 45 DT 40 vs. 3% CT 30 DT 40	1.967	2	0.305	No

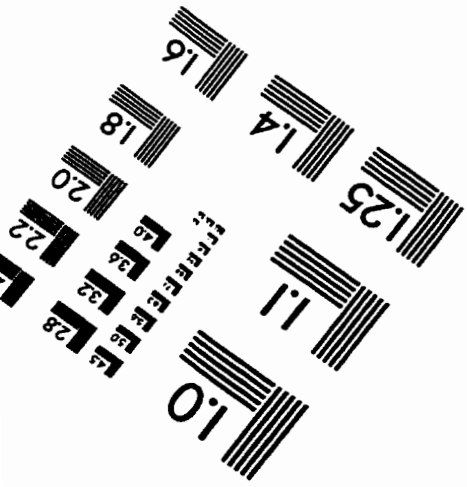
IMAGE EVALUATION
TEST TARGET (QA-3)

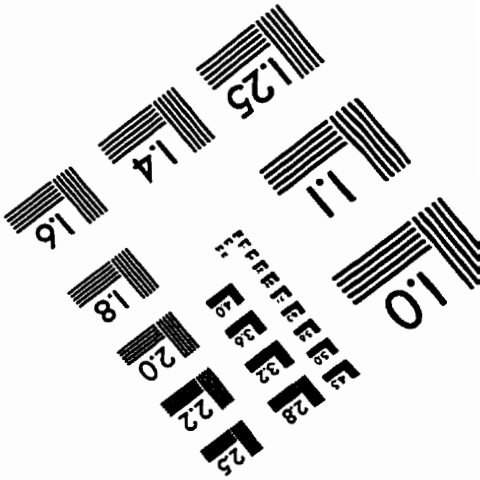












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