AN ABSTRACT OF THE DISSERTATION OF

Angkana Tantituvanont for the degree of Doctor of Philosophy in Pharmacy presented on May 7, 2003

Title: Novel Formulation: Development of Oral Microparticulate Non-Viral DNA Vaccine Delivery System against Infectious Hematopoetic Necrosis Virus (IHNV) in Rainbow Trout, Statistical Design in Matrix Tablets Formulation.

Abstract approved

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J.Mark Christensen

This dissertation describes two different projects. The first is the development of an oral DNA vaccine delivery system for fish. A novel oral DNA vaccine delivery system was developed for Rainbow Trout by combining non-viral vectors (polycationic liposomes or polycationic polymer) to facilitate the DNA vaccine's uptake by cell membranes along with enteric-coated protection of the DNA embedded in microparticles to prevent DNA degradation in the gastrointestinal tract. Spray drying and spray coating bead techniques were employed in the preparation of the DNA vaccine microparticles. The spray drying technique allowed production of spherical shape enteric-coated microparticles with a particle size range of 0.18 to 20 μm. Larger particle sizes of 40-50 mesh were obtained from the spray-coated bead technique. The resultant DNA vaccine microparticles were granulated with regular

fish feed and given to fish to investigate the efficacy of the delivery system in providing protection against IHNV, and to demonstrate the ease of administration in fish. An *in vivo* fish trial experiment showed improvement in fish survival rate when fish were immunized with larger particle size DNA vaccine microparticles. Further research to find effective vector carriers for the DNA vaccine delivery system and to seek modifications of the delivery system that will still prevent the denaturation of plasmid DNA that will also facilitate membrane uptake of the DNA vaccine is needed in order to develop a safe, effective, and commercially viable vaccine to control the outbreak of IHNV

The second project of the dissertation is prediction of *in vitro* drug release profiles from a novel matrix tablet spray-coated with a barrier membrane using mathematical and statistical models. Tablets were prepared by direct compression followed by spray coating. The relationship of the amount of hydrophilic materials in the core tablets and barrier thickness on drug release mechanism was investigated using factorial design and regression analysis. Drug release characteristics were influenced and can be controlled by modifying the amount of hydrophilic materials in the core tablet and the barrier thickness. Mathematical equation generated from regression analysis of n-value, lag time, and percent drug release as a function of the amount of hydrophilic material and the amount of coating material applied can now be used as a tool for predicting and optimizing *in vitro* drug release from matrix tablets spray-coated with a barrier membrane.

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# Novel Formulation: Development of Oral Microparticulate Non-Viral DNA Vaccine Delivery System against Infectious Hematopoetic Necrosis Virus (IHNV) in Rainbow Trout, Statistical Design in Matrix Tablets Formulation.

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APPROVED:

Redacted for privacy

Major Professor, representing Pharmacy

Redacted for privacy

Dean of the College of Pharmacy

Redacted for privacy

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Angkana Tantituvanont, Author

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## CONTRIBUTION OF AUTHORS

Dr. J. Mark Christensen was involved in all aspects of this work. Benjamin E. Simon and Doug Leisy produced the plasmid DNA encoded with luciferase reporter gene and the IHNV glycoprotein. Dr. Jerry R. Heidel was involved in histopathology analysis of fish intestinal tract. Dr. David Birkes was involved in statistical analysis of matrix tablets spray-coated with the barrier membrane.

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# DEDICATION

To my mom

who always reminds me that

life is worth fighting for

# Novel Formulation: Development of Oral Microparticulate Non-Viral DNA Vaccine Delivery System against Infectious Haematopoetic Necrosis Virus (IHNV) in Rainbow Trout, Statistical Design in Matrix Tablets Formulation

## Chapter 1 Introduction

Genetic immunization using naked DNA has been shown to be effective in inducing an immune response against several types of viruses. Presently, vaccination with naked DNA is limited to parenteral administration which is not practical when large numbers of small fish are involved. Oral DNA vaccine delivery is more practical way to mass vaccinate large numbers of fish. Oral vaccination provides advantages over traditional vaccination approaches such as immersion as no stressful handling of fish occur. A greater economical benefit is seen due to less labor costs for vaccination, the ease of administration of the vaccine orally which causes no interference with routine fish husbandry. The difficulty in transportation of plasmid DNA across the cell membrane and the denaturation of DNA by proteolytic gastrointestinal tract enzymes and the acidic environment in the stomach, makes the efficacy of oral vaccination relatively low compared to parenteral injection. Prevention of DNA degradation by incorporating plasmid DNA into enteric-coated polymers allows passage of the intact DNA to the site of cell uptake and enhancing cellular membrane uptake of the DNA with the use of carrier vectors that will subsequently stimulate an immune response.

Chapter 2 presents the formulation development and optimization of an enteric-coated microparticulate oral DNA vaccine. Modified spray drying and solvent evaporation techniques were employed to produce the enteric-coated microparticles. The effect of formulation ingredients on the release of bovine serum albumin and plasmid DNA from enteric-coated microparticles is presented in this chapter. Microparticles characteristics such as production yield, surface morphology, particle size, *in vitro* release profiles, and degree of polymer swelling are also discussed.

Chapter 3 describes the effect of liposomes and cationic polymers as carrier vectors on cell transfection efficiency. Caco-2 and CHSE-214 cells were used as *in vitro* model for the study of gene transfer. Transfection efficiency of these vectors was evaluated with reference to lipid compositions, carrier to DNA charge ratio, and stability of DNA complexes against DNase.

Chapter 4 presents formulation and *in vivo* biostudies of a novel microparticulate oral DNA vaccine against infectious haematopoetic necrosis virus (INHV) in Rainbow trout. Vaccine was prepared by forming the complexation of naked DNA with non-viral vectors followed by incorporating these resulting complexes into enteric-coated microparticles using either a modified spray drying or spray coating technique. The efficacy of the oral IHNV vaccine to provide protection against IHNV is described in detail in this chapter.

Chapter 5 mainly deals with the use of statistical and mathematical tools in generating model equations to predict *in vitro* drug release profiles from matrix tablets

spray-coated with a barrier membrane. Using factorial design and regression analysis approaches, the relationship of the amount of hydrophilic materials in the core tablets and thickness of the spray-coated polymer barrier surrounding the tablet on the drug release characteristics was investigated.

# Chapter 2

## Evaluation and Preparation of Enteric-Coated Property Microparticles by Modified Spray Drying Technique and Solvent Evaporation Technique

**Angkana Tantituvanont and J. Mark Christensen**

## ABSTRACT

The aim of this study was to investigate the potential use of a spray drying technique on the preparation and production of enteric-coated microparticles (ECAMs). Also the operating conditions such as solution feed rate, atomized air pressure, fluidize air, and temperature, were varied to determine their effects on the characteristics of spray-dried microparticles. Microparticles characteristics were studied and evaluated with respect to production yield, surface morphology, particle size, *in vitro* release profiles, degree of polymer swelling, and stability of DNA embedded in microparticles. Enteric-coated microparticles (Eudragit® L-30 D, hydroxypropylmethylcellulose phthalate-55, and cellulose acetate phthalate microparticles) embedded with bovine serum albumin (BSA) or plasmid DNA (pEGFP-N1) were prepared by spray drying method. Spray drying technique allowed the production of spherical shape microparticles with a size range from 0.18 to 20 μm. Spray-dried microparticles possessed a log-normal size distribution or skewed size distribution. *In vitro* drug release profiles revealed that BSA and pEGFP-N1 were protected from an acidic environment for at least 2 hours. The degree of swelling of the microparticles in acidic solution increased with time. The optimal operating conditions for spray drying technique for microparticle production were found to be a solution feed rate of 30 ml/hr, an atomized air pressure of 15 psi, air pressure at 50 percent of full capacity from the fluidized bed unit, and the inlet and outlet drying temperatures set at 60 °C and 40 °C, respectively. The optimum storage condition for

the spray-dried microparticles was found to be 4 °C in desiccators. Light did not affect the stability of pEGFP-N1.

# INTRODUCTION

Most infectious pathogens enter the body through mucosal surfaces of the gastrointestinal, nasopharyngeal, pulmonary, and genitourinary tract. Effective gastrointestinal (GI) mucosal delivery of antigens are extremely desirable since immunization at these surfaces offers a potent mechanism in defending against pathogen entry at the site of entry (Aramaki et al., 1993). There are other advantages to deliver antigens directly to site of entry over the traditional methods of vaccination, such as easy administration, no stressful handling, and reduced costs to prepare, administer, and store the vaccine thereby yielding a less economic impact. Vaccines currently available are delivered mainly by subcutaneous, intramuscular, and intradermal injection route. These routes of administration may not activate mucosal immune system resulting in a lack of effective mucosal immunization (Gupta et al., 1998). Mucosal immunity is often the initial obstacle offending organisms encounter and it may be more advantageous to stimulate mucosal immunity than systemic immunity during vaccination to protect against virulent microorganisms. The major difficulties in developing potential GI mucosal vaccines are instability of DNA/protein/peptide antigens caused by hydrolytic/proteolytic enzymes (Li et al., 1998) and environment in the gastrointestinal tract (GUT), where induction of tolerance to soluble antigens, and inefficient uptake of antigens by mucosal surfaces often occurs (Gupta et al., 1998). Incorporating antigens into gastric resistant polymers such as cellulose acetate phthalate (CAP), hydroxypropyl methylcellulose phthalate (HPMCP-55), and Eudragit® L-30 D, has provided protection of antigens

against proteases and the acidic environment in the GUT. This protection of the antigen allows effective transport of bioactive antigens to the lymphatic system of the GI tract and subsequently inducing a mucosal immune response.

In the past few years, many studies have focused on the use of biodegradable microparticles, especially PLGA for effective mucosal immunization in animals (Gupta et al., 1998). It was reported that protein antigens encapsulated in biodegradable microparticles were successfully delivered to M cells without the induction of tolerance and subsequently stimulated the mucosal immune response (Gupta et al., 1998).

This finding as well as the ability of gastric resistant polymers to protect the antigen against the harsh environment in the GUT makes enteric-coated microparticles a viable candidate for a mucosal delivery system of antigens.

A variety of procedures have been used in the production of microparticles, coacervation-phase separation, solvent evaporation, spray drying, spray congealing, spray embedding, and spray polycondensation. In the present work, spray drying and solvent evaporation technique were selected to prepare enteric-coated microparticles.

Spray drying has been used widely in a variety of fields such as chemical, biochemical, pharmaceutical, and food industries (Conte et al., 1994; Freitas and Muller, 1998). It is commonly used in the preparation of milk, eggs, proteins, ceramics, and fertilizers. Spray drying is a one step process in converting the liquid droplets into dry products (Freitas and Muller, 1998). The operation consists of four steps: atomization of the feed solution into a spray, spray-air contact, drying of the

feed, and product collection (Freitas and Muller, 1998; Maa et al., 1998; Master, 1985). Different types of atomization are available and classified by the design of nozzle such as rotary atomization, pressure atomization, and two-fluid (pneumatic) atomization (Broadhead et al., 1992). In this study, a two-fluid nozzle was used in the preparation of microparticles. This atomization system allows the feed solution and air pressure to individually pass through the nozzle where they combine followed by the atomization of the feed solution into spray (Broadhead et al., 1992). A variety of designs of spray dryers as co-current manner, counter current manner and intermediate between co- and counter- current are also available. For heat sensitive materials such as protein/peptide drugs, a spray dryer with co-current manner is preferred as the spray-dried product is in contact only with the cool air (Conte et al., 1994; Freitas and Muller, 1998). Throughout the spray drying process, the temperature of the dried product is considerably lower than the outlet temperature due to a high moisture evaporation rate, which consequently prevents the degradation of heat sensitive materials as well as minimizing the loss of biological activity (Broadhead et al., 1992; Freitas and Muller, 1998). Generally, with the co-current dryer, the maximum temperature of the dried product is approximately 15 °C - 20 °C below the outlet temperature (Freitas and Muller, 1998). In contrast, with a counter-current dryer, the spray-dried product is subjected to the hottest airflow. Co-current spray dryer was used in the preparation of microparticles in this study.

The aim of this study was to investigate the potential use of a spray drying technique in the preparation of enteric-coated microparticles. The effect of operating

conditions to produce enteric-coated microparticles as well as the type of polymers used to create the microparticles were studied and evaluated to determine the characteristics of the ECAMs with reference to production yield, surface morphology, particles size, and *in vitro* drug release profiles.

# MATERIALS AND METHOD

## Polymers and Additives

The acrylic resins Eudragit® L-30 D and Eudragit® L100-55, gifts from Degussa Rohm Pharma Polymers, Degussa Corporation (Piscataway, NJ, USA), hydroxypropylmethylcellulose phthalate (HPMCP-55), and cellulose acetate phthalate (CAP) from Eastman Chemical Company (Kingsport, TN, USA) were used as enteric-coated polymers for microparticle preparation. Tween 20 as a dispersing agent, triethyl citrate as plasticizer (TEC), ammonium hydroxide ($NH_4OH$) and sodium hydroxide (NaOH) for pH adjustment were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

## Model Drugs

Bovine serum albumin (BSA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and plasmid DNA (pEGFP-N1), Figure 2.1, was a gift from Dr. Joann Leong, Department of Microbiology, Oregon State University (Corvallis, OR, USA).

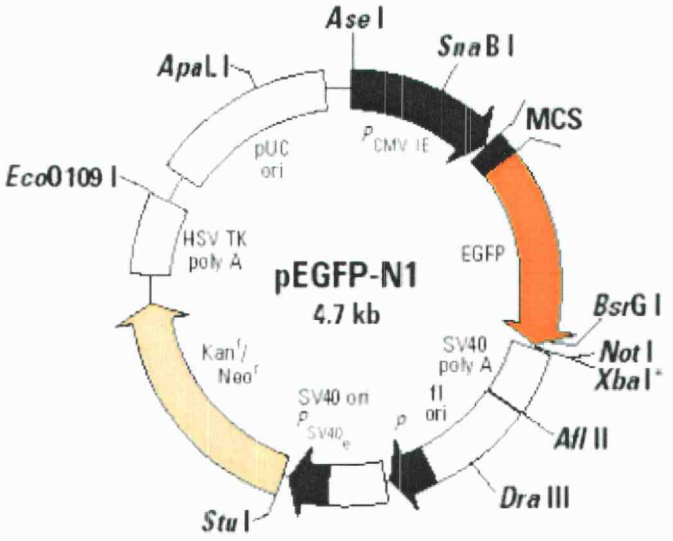



**Figure 2.1: Restriction map of pEGFP-N1 (http://www.clontech.com/techinfo/vectors/vectorsE/pEGFP-N1.shtml)**

## Cell Culture

Human adenocarcinoma cells (Caco-2 cells) were maintained in Dulbecco's modified eagle medium (DMEM), Life Technologies Inc. (Rockville, Maryland, USA) supplemented with 10% fetal bovine serum (FBS), SUMMIT Biotechnology (Fort Collins, CO, USA), 1% MEM non-essential amino acid solution, Life Technologies Inc. (Rockville, Maryland, USA), 1% of 100 nM sodium pyruvate, Sigma Chemical Co. (St. Louis, MO, USA), and 10,000 unit penicillin, 10 mg streptomycin, and 25 µg amphotericin B per ml in 0.9% sodium chloride, Sigma Chemical Co. (St. Louis, MO, USA ) in a 1:100 ratio of antibiotic antimycotic solution to medium. The pH of medium was maintained at 7.4 by buffering with sterile sodium bicarbonate solution,

Sigma Chemical Co. (St. Louis, MO, USA). Caco-2 cells were grown and maintained in T 75 $cm^2$ tissue culture flasks at 37 °C in 5% $CO_2$ humidified atmosphere.

**Methods**

Two different techniques, spray drying and solvent evaporation technique were used to produce microparticles. Microparticles obtained from spray drying techniques involved using two different machines, Yamato spray dryer (Yamato Pulvis Basic Unit Model GB-21, Yamato Scientific Co., Ltd, Tokyo, Japan) and a modified laboratory fluid bed spray dryer unit developed at Oregon State University in the Pharmaceutical laboratory. The effect of operating conditions such as solution feed rate, atomized air pressure, inlet-outlet temperature as well as the potential of both machines on microparticle production and characteristics were studied as followed.

**Preparation of Polymer Solution**

The enteric-coated polymers were suspended in 50 ml sterile deionized (DI) water and magnetically stirred at 100 rpm for 15 minutes. The pH of the suspension was adjusted to the about 7.4 with ammonium hydroxide or sodium hydroxide for completely dissolving of polymer. Plasticizer (TEC) was added into polymer solution with another 15 min stirring. BSA or p-EGFP-N1 was solubilized in sterile deionized water (DI water) and added into polymer solution with continuous stirring. The final

volume of polymer solution was adjusted to 100 ml with sterile DI water. The obtained solution was mixed thoroughly for 20 min using magnetic stirrer.

**Preparation of Microparticles by Spray Drying Technique**

Embedding of BSA or pEGFP-N1 into the enteric-coated microparticles was performed based upon formulations and techniques listed in Table 2.1. Yamato spray dryer (Machine 1) and a modified laboratory fluid bed spray dryer unit (Machine 2) were used to produce microparticles.

**Table 2.1: Ingredients used in microparticles formulations[a]**

| Formulation | Eudragit® L-30 D (g) | HPMCP-55 (g) | CAP (g) | TEC (g) | BSA (g) | pEGFP-N1 (mg) | pH adjusting agents | Machine |
|---|---|---|---|---|---|---|---|---|
| 1 | 28 | - | - | 2.8 | 1.0 | - | $NH_4OH$ | 1 |
| 2 | 14 | - | - | 1.4 | 1.0 | - | $NH_4OH$ | 1 |
| 3 | 10.5 | - | - | 1.05 | 1.0 | - | $NH_4OH$ | 1 |
| 4 | 7 | - | - | 0.7 | 1.0 | - | $NH_4OH$ | 1 |
| 5 | 6.125 | - | - | 0.6125 | 1.0 | - | $NH_4OH$ | 1 |
| 6 | 5.25 | - | - | 0.525 | 1.0 | - | $NH_4OH$ | 1 |
| 7 | 4.375 | - | - | 0.4375 | 1.0 | - | $NH_4OH$ | 1 |
| 8 | 3.5 | - | - | 0.35 | 1.0 | - | $NH_4OH$ | 1 |
| 9 | 6 | - | - | 0.6 | 2.5 | - | $NH_4OH$ | 2 |
| 10 | 6.5 | - | - | 0.65 | 2.0 | - | $NH_4OH$ | 2 |

[a]HPMCP-55 represents hydroxypropylmethylcellulose phthalate, CAP represents cellulose acetate phthalate, TEC represents triethyl citrate, BSA represents bovine serum albumin, pEGFP-N1 is a plasmid DNA, $NH_4OH$ represents ammonium hydroxide, and NaOH represents sodium hydroxide. Machine 1 is a Yamato spray dryer. Machine 2 is a modified laboratory fluid bed spray dryer unit.

**Table 2.1: Ingredients used in microparticles formulations[a] (continued)**

| Formulation | Eudragit® L-30 D (g) | HPMCP-55 (g) | CAP (g) | TEC (g) | BSA (g) | pEGFP-N1 (mg) | pH adjusting agents | Machine |
|---|---|---|---|---|---|---|---|---|
| 11 | 7 | - | - | 0.7 | 1.5 | - | $NH_4OH$ | 2 |
| 12 | 7.5 | - | - | 0.75 | 1.0 | - | $NH_4OH$ | 2 |
| 13 | - | 7 | - | 0.7 | 1.5 | - | $NH_4OH$ | 2 |
| 14 | - | - | 7 | 0.7 | 1.5 | - | $NH_4OH$ | 2 |
| 15 | 7 | - | - | 0.7 | - | 2 | $NH_4OH$ | 2 |
| 16 | - | 7 | - | 0.7 | - | 2 | $NH_4OH$ | 2 |
| 17 | - | - | 7 | 0.7 | - | 2 | $NH_4OH$ | 2 |
| 18 | 7 | - | - | 0.7 | 1.5 | - | NaOH | 2 |

[a]HPMCP-55 represents hydroxypropylmethylcellulose phthalate, CAP represents cellulose acetate phthalate, TEC represents triethyl citrate, BSA represents bovine serum albumin, pEGFP-N1 is a plasmid DNA, $NH_4OH$ represents ammonium hydroxide, and NaOH represents sodium hydroxide. Machine 1 is a Yamato spray dryer. Machine 2 is a modified laboratory fluid bed spray dryer unit.

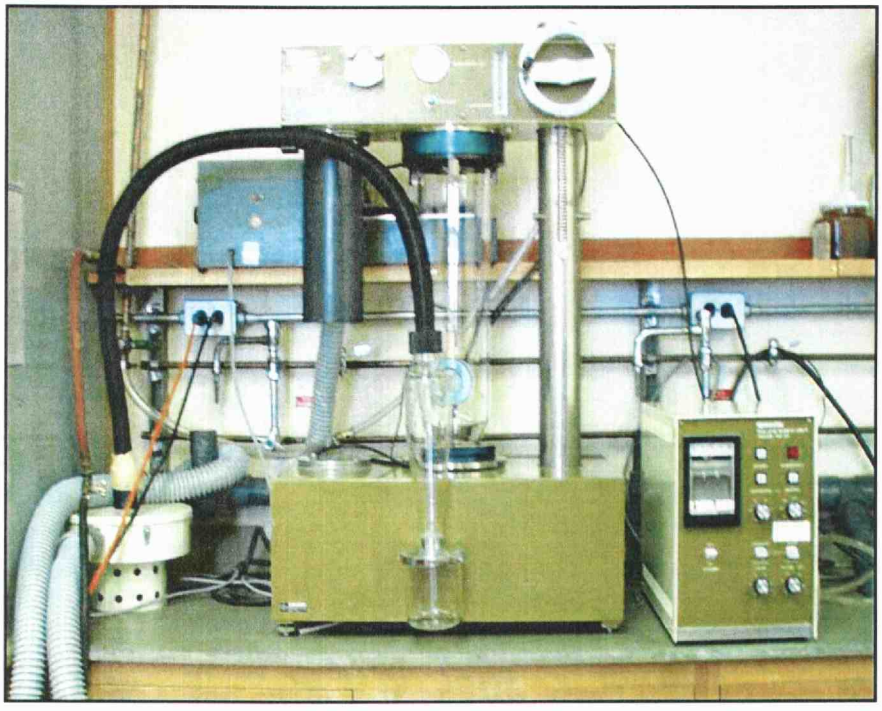
## Microparticle Preparation with Yamato Spray Dryer

Polymer solution loaded with BSA prepared according to formulations listed in Table 2.1 was fed along a needle rod using a peristaltic pump and was atomized into droplets by compressed air in the spray dryer chamber. A flow of heated air aspirated in a spray dryer chamber induced the evaporation of solvent from the droplets leading to the formation of solid microparticles (Esposito et al., 2000). The resultant particles were separated from the exhaust air in a cyclone and settled in a collector (Figure 2.2).

## Influence of Solution Viscosity on the Microparticle Preparation and Characteristics

To determine the optimal viscosity of polymer solution for microparticle preparation, different solution viscosities were evaluated. Microparticles were produced using a polymer solution containing Eudragit® L-30 D by varying the amount of Eudragit® L-30 D in the following fashion, 28, 14, 10.5, 7, 6.125, 5.25, 4.375, and 3.5 g per 100 ml deionized water, differing viscosity solution of microparticles were produced using the Yamato spray dryer. Polymer solution viscosity was measured using Brookfield viscometer model LV-T, Brookfiled Engineering Laboratories, Inc. (Stoughton, MA, USA). The viscosity of polymer solution that provides the least formation of film and fiber was chosen for further study.

A



B

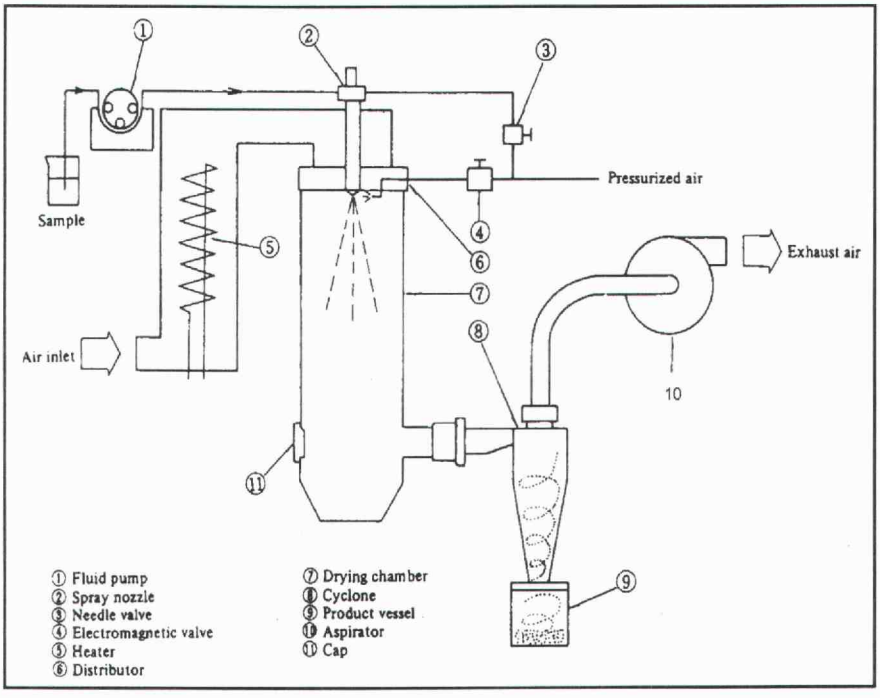



**Figure 2.2: Yamato spray dryer.** Panel A and Panel B shows a picture and a schematic diagram of Yamato spray dryer, respectively.

**Microparticle Preparation with Modified Laboratory Fluid Bed Spray Dryer**

Polymer solution loaded with either BSA or pEGFP-N1 produced according to formulations listed in Table 2.1 was atomized through a 0.5 mm spray nozzle with a constant feed rate from a peristaltic pump, Rainin Instrument Co. Inc. (Woburn, MA, USA). Atomized air pressure was set at approximately 50% of full air capacity in the fluidized bed unit. The inlet and outlet drying temperatures were varied depending upon the experiment. The solid microparticles were obtained at the top of spray dry chamber (Figure 2.3).

A

B

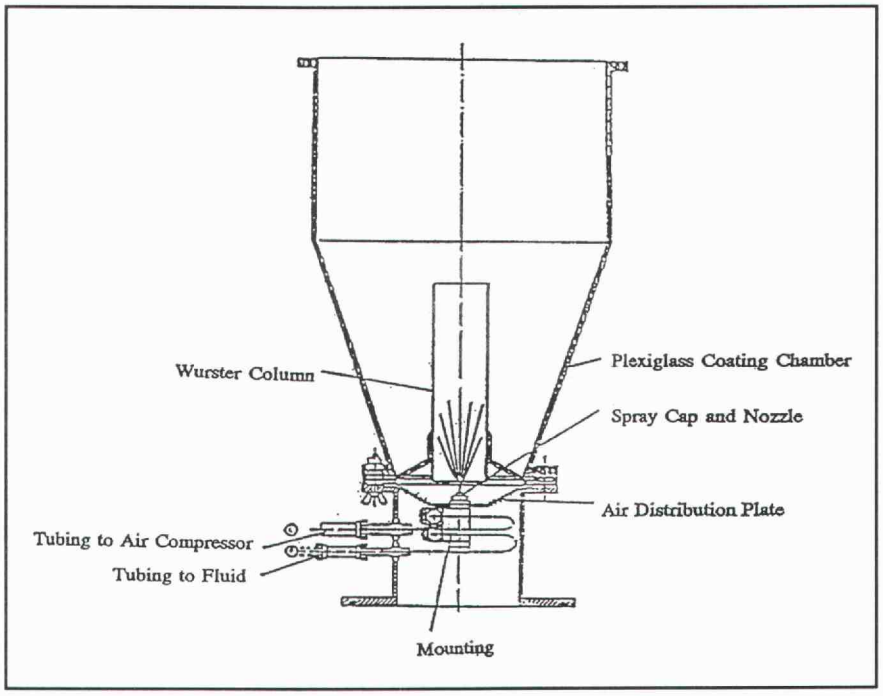



**Figure 2.3: Modified laboratory fluid bed spray dryer.** Panel A and Panel B shows a picture and a schematic diagram of a modified laboratory fluid bed spray dryer.

**Influence of Operating Conditions on Microparticle Recovery and Characteristics**

To examine the effect of operating conditions on microparticle recovery and characteristics, the spray drying operating parameters were varied based upon the list in Table 2.2 for Yamato spray dryer. In the preparation of microparticles using modified laboratory fluid bed spray dryer, the operating parameters were changed one by one at different levels (Table 2.3). The following parameters were investigated: (a) solution feed rate of 25, 30, 35, and 45 ml/hr; (b) atomized air pressure of 5, 10, 15, and 20 psi; (c) drying-air temperature (inlet-outlet) of 30-10, 40-20, 50-30, 60-40 °C.

**Table 2.2: Operating conditions for microparticles prepared by Yamato spray dryer**

| Compressed Air | Solution Feed Rate (ml/min) | Outlet Temperature (°C) | Inlet Temperature (°C) |
|---|---|---|---|
| 50% of full capacity | 0.5 | 120 | 100 |
| | | 100 | 80 |
| | | 80 | 60 |
| 100% of full capacity | 0.5 | 120 | 100 |
| | | 100 | 80 |
| | | 80 | 60 |

**Table 2.3: Operating conditions for microparticles prepared by modified laboratory fluid bed spray dryer**

| Factors | Level |
|---|---|
| Solution Feed Rate (ml/hr) | 25<br>30<br>35<br>45 |
| Atomized Air Pressure (psi) | 5<br>10<br>15<br>20 |
| Drying Air Temperature (°C) (inlet-outlet) | 30-10<br>40-20<br>50-30<br>60-40 |
| **Optimal Conditions** | |
| **Factor** | **Level** |
| Solution Feed Rate (ml/hr) | 30 |
| Atomized Air Pressure (psi) | 15 |
| Drying Air Temperature °C (inlet-outlet) | 60-40 |
| Fluidized Air Pressure | 50% of full capacity |

**Percent Recovery**

Microparticle recovery efficiencies were determined as percentage of weight of the recovered spray-dried microparticles with respected to the total amount of solid used in the preparation (equation 1).

$$\text{Percent Recovery} = \frac{W}{T} \times 100 \qquad \text{equation (1)}$$

Where: W = Weight of the recovered spray-dried microparticles

T = Total amount of solid utilized

**Optimization of Drug-Polymer Ratio**

To obtain the optimal formulation for microparticle preparation, different amounts of polymer-drug ratio were investigated. Microparticles were produced with Eudragit® L-30 D and BSA in varying polymer-drug ratio of 6:2.5, 6.5:2, 7:1.5, 1.5:1 (see formulation 9-12 in Table 2.1) using modified laboratory fluid bed spray dryer. The operating parameters were 30 ml/hr solution feed rate, 15 psi atomized air pressure, 60-40 °C inlet-outlet drying air temperature, and 50% of full capacity of fluidize air pressure. The optimal formulation was determined with respect to the percent entrapment efficiency calculated from percentage of BSA release from microparticles during 2 hours in simulated gastric fluid (SGF) pH 1.23 followed by 2

hours in simulated intestinal fluid (SIF) pH 7.4. The formulation that had the best percent entrapment efficiency was chosen for further study.

## Influence of Polymer Type on *in vitro* Release Profiles of BSA from Microparticles

To determine the influence of polymer type on *in vitro* release profiles of BSA from microparticles, enteric-coated property polymers, namely Eudragit® L-30 D, HPMCP-55, and CAP were employed in microparticle preparation. Microparticles were produced according to formulation 11, 13, 14, 15, 16, and 17 using modified laboratory fluid bed spray dryer (Table 2.1).

## Preparation of Microparticles by Solvent Evaporation Technique

Microparticles loaded BSA or pEGFP-N1 was prepared with three different enteric-coated polymers, namely Eudragit® L-30 D, HPMCP-55, and CAP. Microparticles were produced according to formulation listed in Table 2.4.

**Table 2.4: Formulation for microparticles prepared by solvent evaporation technique**

| Ingredients | Formulation | |
|---|---|---|
| | 19 | 20 |
| Enteric-coated polymer (g) | 0.1 | 0.1 |
| Water (ml, aqueous phase) | 40 | 40 |
| Acetone (ml, organic phase) | 5 | 5 |
| Tween 20 (gm, stabilizer) | 0.3 | 0.3 |
| Solution pH (adjusted by hydrochloric acid) | 3.5 | 3.5 |
| BSA (mg) | 10 | - |
| pEGFP-N1 (mg) | - | 0.5 |

The enteric-coated polymer in the amount of 0.1 g was dissolved in acetone (organic phase) while BSA (10 mg) or pEGFP-N1 (0.5 mg) was dissolved in sterile DI water pH 3.5 (aqueous phase) containing Tween 20 as a dispersing agent. The aqueous phase containing model drug (BSA or pEGFP-N1) was then added into the organic phase containing enteric-coated polymer and the mixture of the two solutions was stirred at 600 rpm for at least 8 hours to ensure a complete evaporation of acetone, leading to the formation of solid microparticles. The resulted microparticle suspension was centrifuged at 15,000 rpm (31,000 ×g) for 30 minutes using high speed centrifuge Beckman JS-HS, Beackman Instrument Inc. (Pala Alto, CA, USA) and the supernatant was discarded. Microparticles were washed twice with sterile DI water pH 3.5 followed by vortexing and centrifugation to remove the unencapsulated

drug. Microparticles were collected and resuspended in sterile DI water (pH 3.5) for further use.

## Analysis of BSA and pEGFP-N1 Extracted from Enteric-coated Microparticles

### *In vitro* Release of BSA from the Microparticles

For spray-dried BSA microparticles, the dissolution test was performed in triplicate with the USP apparatus II dissolution machine, Vankel Industries, Inc. (Edison, NJ, USA). The dissolution of 0.5 g of spray-dried BSA loaded microparticles was carried out first in 750 ml simulated gastric fluid pH 1.23 for 2 hours followed by 2 hours in 1000 ml simulated intestinal fluid pH 7.4. The paddle speed was set at 100 rpm with the bath temperature set at 37.5 °C. Samples of 3 ml were collected at 5, 10, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 180, 195, 210, 225, and 240 minutes. For the microparticles prepared by the solvent evaporation technique, the dissolution test was performed in triplicate with the USP apparatus III dissolution machine, BIO-DIS extended release tester, Vankel Industries, Inc. (Edison, NJ, USA). Approximately 10 ml of suspension microparticles containing about 0.45 g of BSA loaded microparticles was carried out first in 175 ml simulated gastric fluid pH 1.23 for 2 hours followed by 2 hours in 250 ml simulated intestinal fluid pH 7.4. About 2 ml of solution was taken at 5, 10, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 195, 210, 225, and 240 minutes. BSA released from microparticles was

determined by Lowry's protein assay using Beckman UV spectrophotometer at a wavelength of 540 nm.

**Reagents for Lowry's Protein Assay**

Reagent A1, 2% sodium tartrate, was prepared by dissolving 2.0 g of sodium tartrate, $Na_2C_4H_4O_6$ (Sigma Chemical Co., St. Louis, MO, USA), in 100 ml of deionized water. Reagent $A_2$, 1% copper sulfate, was prepared by dissolving 1.0 g of copper sulfate, $CuSO_4.H_2O$ (Sigma Chemical Co., St. Louis, MO, USA), in 100 ml of deionized water. Reagent $A_3$, 2% sodium carbonate, was prepared by dissolving 20.0 g of sodium carbonate, $Na_2CO_3$ (Sigma Chemical Co., St. Louis, MO, USA), in 1 liter of 0.1 N sodium hydroxide, NaOH (Sigma Chemical Co., St. Louis, MO, USA). Reagent A was prepared by mixing reagents $A_1$, $A_2$, $A_3$ in the ratio of 0.75:0.75:15. Reagent B was prepared by adding 5 ml of 2 N Folin & Ciocalteu's phenol reagent (Sigma Chemical Co., St. Louis, MO, USA) into 50 ml of deionized water.

**Lowry's Protein Assay**

To 1 ml of dissolution sample containing BSA, 1 ml of reagent A was added. The mixture was mixed well and allowed to stand for 15 minutes at room temperature. After 15 minutes, 3 ml of reagent B was added and mixed very rapidly. The mixture was allowed to stand for another 45 minutes at room temperature. The absorbance of

the solution was measured at the wavelength of 540 nm using a spectrophotometer, Beckman DU-600, Beckman Coulter, Inc. (Fullerton, CA, USA).

### *In vitro* Release of pEGFP-N1 from the Microparticles

The release studies of pEGFP-N1 entrapped in the microparticles were performed in triplicate. Briefly, 20 mg of microparticles loaded with pEGFP-N1 were incubated in 1 ml of sterile simulated gastric fluid pH 1.23 for 2 hours followed by 2 hours in 1 ml of sterile simulated intestinal fluid pH 7.4. Samples of 0.8 ml were removed at 15, 30, 60, 120, 130, 140, 150, 180, 240 minutes and centrifuged for 1 minute at 15,000 rpm (12,576 ×g) using Eppendorf Centrifuge 5415C, Brinkmann Instruments, Inc. (Westbury, NY, USA). The supernatant containing pEGFP-N1 was taken for DNA purification using QIAquick PCR purification kit, QIAGEN Inc. (Valencia, CA, USA). The recovered pEGFP-N1 was determined by the absorbance obtained at the wavelength of 260 nm in the spectrophotometer, Beckman UV spectrophotometer, Beckman DU-600, Beckman Coulter, Inc. (Fullerton, CA, USA).

### Microparticle Encapsulation Efficiency Determination

Percent encapsulation efficiency (equation 2) was calculated as actual drug loaded in microparticles with respect to theoretical drug loaded.

$$\% \text{ Encapsulation Efficiency} = \frac{L_A}{L_T} \times 100 \qquad \text{equation (2)}$$

Where: $L_A$ = Actual drug load

$L_T$ = Theoretical drug load

**BSA Loaded in Microparticles**

The amount of BSA loaded in microparticles was determined using Lowry's protein assay. In microparticles prepared by the spray drying technique, BSA loaded in microparticles was obtained from the different in the amount of BSA release from 0.5 g of microparticles during 2 hours in simulated gastric fluid (SGF) pH 1.23 and from the same microparticles during 2 hours in simulated intestinal fluid (SIF) pH 7.4.

In solvent evaporation technique of microparticles, 1 ml of the suspended microparticles were centrifuged at 15,000 rpm (12,576 ×g) for 1 minute using Eppendorf Centrifuge 5415C, Brinkmann Instruments, Inc. (Westbury, NY, USA) and the supernatant was discarded. The above procedure was repeated twice with 1 ml sterile DI water pH 3.5 in order to get rid of the excess BSA. The resultant microparticles were dissolved in sterile phosphate buffer pH 7.4 and the amount of encapsulated BSA was measured at 540 nm using Beckman UV spectrophotometer.

**Plasmid DNA (pEGFP-N1) Loaded in Microparticles**

In microparticles prepared by the spray drying technique, pEGFP-N1 was extracted from 20 mg microparticles by dissolving dried microparticles in 1 ml of sterile simulated intestinal fluid pH 7.4. The procedure for extracting the encapsulated plasmid DNA (pEGFP-N1) from the microparticles prepared from solvent evaporation technique was the same as was performed for BSA, previously described. To determine the amount of pEGFP-N1 loaded in microparticles, the extracted plasmid DNA was further purified by QIAquick PCR purification kit followed by measuring the UV absorption at 260 nm using Beckman UV spectrophotometer.

**Analysis of Structural and Functional Integrity of Plasmid DNA**

Structural and functional integrity of extracted plasmid DNA was determined by agarose gel electrophoresis and transfection assay with Caco-2 cells, respectively.

**Agarose Gel Electrophoresis**

Thirty milligrams of spray-dried microparticles corresponding to 1-1.5 μg, pEGFP-N1 were dissolved first with sterile phosphate buffer (pH 7) followed by DNA purification using QIAquick PCR purification kit. The extracted plasmid was mixed with 5 μl gel loading buffer (bromophenol blue 0.05% w/v, sodium dodecyl sulphate 0.05% w/v, sucrose 40% w/v EDTA 0.1 M, pH 8) and subjected to agarose gel

electrophoresis in the presence of ethidium bromide for 10 minutes at 210 Volt. DNA visualization of the gels was carried out under UV lamp, Fotodyne (New Berlin, WA, USA).

**Transfection of Caco-2 cells**

Caco-2 cells ($1 \times 10^6$ cell/ml) were seeded in 12 well plates, and allowed to attach to the plate surface by growing in DMEM supplemented with 10% FBS for 20-24 hours at 37 °C in 5% $CO_2$ humidified atmosphere. Cells were transfected according to the protocol provided with the commercial transfecting reagent, Lipofectin®reagent, Life Technologies, Inc. (Rockville, Maryland, USA). Briefly, cells with 60-70% confluence were washed twice with serum free medium (DMEM supplemented with 1% MEM non-essential amino acid solution, 1% sodium pyruvate) and incubated with plasmid DNA (pEGFP-N1), both unencapsulated and extracted plasmid DNA, for 3 hours at 37 °C in 5% $CO_2$ humidified atmosphere. After 3 hours, the transfection medium was removed and replaced with complete DMEM (DMEM supplemented with 10% FBS, 1% MEM non-essential amino acid solution, 1% sodium pyruvate, and 10,000 unit penicillin, 10 mg streptomycin, and 25 μg amphotericin B per ml in 0.9% sodium chloride in a 1:100 ratio of antibiotic antimycotic solution to medium). Gene expression in Caco-2 cells was observed at 72 hours post transfection by examining the green fluorescence of cells under a fluorescence microscope, Barlets & Stout, Inc. (Bellevue, WA, USA).

## Microparticles Dimension and Morphology Analysis

Microparticles size and size distribution were evaluated by examining the diameter of microparticles on digital microphotographs using inverted optical microscope, Barlets & Stout, Inc. (Bellevue, WA, USA) connected with the digital camera, Diagnostic Instruments Inc. (Burlingame, CA, USA). Size distribution analysis was performed by computerized size analysis system, Image-Pro® Plus version 4.1 for windows (Silver Spring, MD,USA) with at least 1000 microparticles for each sample, after suspending the microparticles in mineral oil containing 1% span 80 as a dispersing agent.

Microparticles surface morphology was determined using scanning electron microscope, AmRay, model 3300FE, AmRay Corp. (Bedford, Mass. USA) performed by Soeldner, A., Department of Botany and Plant Pathology at Oregon State University. Spray-dried microparticles were analyzed at 7 kV after metallization with gold coating (Soeldner, A., personal communication).

## Influence of pH Adjusting Agents on the Release Profile and Morphology of Eudragit® L-30 D Microparticles

Two different pH adjusting agents, sodium hydroxide and ammonium hydroxide, were used to investigate the effect of the pH adjusting agent on *in vitro* drug release from microparticles and microparticle morphology characteristics.

Microparticles were produced according to formulations 11 and 18 using modified laboratory fluid bed spray dryer (Table 2.1).

**The Degree of Swelling of Eudragit® L-30 D Microparticles**

Spray-dried microparticles (30 mg) were suspended in 2 ml of simulated gastric fluid using 12 well culture plates. Degree of swelling of microparticles was determined by examining the change in the diameter of microparticles from the digital microphotographs at 15, 30, 45, 60, and 120 minutes. The size analysis was performed by the computerized analysis system as described previously.

**Effect of Storage Condition on Plasmid DNA Integrity over Time Period**

Microparticles embedded with pEGFP-N1 were stored at room temperature, 4 °C, and -20 °C in both light and dark conditions and the structural integrity of pEGFP-N1 was determined using agarose gel electrophoresis immediately after their preparation and 6 months later.

***In vitro* Uptake of Microparticles by Caco-2 cells**

The procedure for transfecting cells was performed as previously described. Briefly, Caco-2 cells were seeded in the amount of $1 \times 10^6$ cell per well in a 12 well culture plate and allowed to grow in DMEM supplement with 10% FBS at 37 °C in

5% $CO_2$ humidified atmosphere before being treated with microparticle formulations. The experiments were carried out with two different formulations, spray-dried microparticles and suspension microparticles. In the spray-dried microparticles formulation, Caco-2 cells were treated with 1, 5, 10, and 100 mg of microparticles corresponding to 4.9, 24.5, 49, and 490 ng of plasmid DNA (pEGFP-N1) and incubated for 2 to 4 hours in serum free medium at pH 5.3 and 7.4. For the suspension formulation, two sets of experiments were performed. First, Caco-2 cells were treated with microparticles obtained from a 2.5 mg particles/ml stock preparation of suspended microparticles. The quantity of 2.5, 25, 250, 500, 750, 1250, 2500, and 3750 μg of microparticles which corresponds to 6.25, 62.5, 625, 1250, 1875, 3125, 6250, 9375 ng of plasmid DNA (pEGFP-N1) were incubated for 2 to 4 hours in serum free medium at pH 5.3 and 7.4. Second, Caco-2 cells were treated with 250, 500, 750, 1000, 2500 μg of microparticles corresponding to 1.323, 2.646, 9.969, 5.25, 13.125 μg of plasmid DNA (pEGFP-N1) for 2 to 24 hours in serum free medium at pH 5.3 and 7.4. Caco-2 cells treated with the complex of Lipofectin® mixed with pEGFP-N1 and untreated cells served as a positive control and negative control, respectively. At the end of incubation period, cells were washed twice with phosphate buffer saline (PBS) and maintained in complete medium. After 72 hours, gene expression in Caco-2 cells was observed by examining the expression of green fluorescence in cells under the fluorescence microscope.

# RESULTS AND DISCUSSION

## Preparation of Microparticles by Yamato Spray Dryer

### Influence of the Operation Conditions and Solution Viscosity on the Microparticles Preparation and Characteristics

Initial attempts to produce microparticles with the Yamato spray dryer did not give a good yield because most of the polymer solution formed a thin film around the drying chamber's wall. Factors that have an influence on the microparticle preparation are solution feed rate, compressed air pressure entering the chamber, and drying temperature. In order to get solid particles and avoid getting the thin film forming on the drying chamber wall, the droplets of polymer solution need to dry before hitting the chamber's wall. To do this, the air aspirated in the chamber as well as the inlet temperature must be high enough to evaporate away all the solvent (Freitas and Muller, 1998). Raising the air aspirator to a high power also leads to a low yield as the particles have a tendency to be blown out of the exhaust flume (Freitas and Muller, 1998)

Another major problem was the formation of fibers, which is caused by insufficient forces to break the liquid filament into droplets. This characteristic depends strongly on the property of polymer and the viscosity of polymer solution. In

general, fewer fibers are obtained with lower viscosity polymer solutions (Bodmeier and Chen, 1988).

"Strong intermolecular bonds with adhering stiffness of the polymeric chains lead to a strong chain interaction and to a high degree of the fiber formation" (Bodmeier and Chen, 1988).

Decreasing the viscosity of polymer solution from 335 centipoises (mPa·S) to 57.5 centipoises (mPa·S) subsequently reduced the presence of the thin film and fiber formation in half but there was no improvement in the percent yield, Table 2.5. Pavanetto et al. (1993) reported that the polymer filaments formation on the spray chamber's wall were the result of high polymer concentration and the low yield of microparticles was caused by the high loss of microparticles in the spray dryer apparatus due to low polymer concentration. Film breaking was observed with a further decrease in solution viscosity. Yamoto spray dryer provided relatively low yield of microparticles. Less than 2% yield was observed along with extensive film and fiber formation. Thus to improve the yield and minimize the formation of film and fibers, microparticle preparation with the modified laboratory fluid bed spray dryer was performed.

**Table 2.5: Influence of viscosity on the microparticle preparation and characteristics**

| Formulation | Eudragit® L-30 D (g) | Viscosity (Centipoises, mPa.S) | TEC (g) | BSA (g) | Fiber and Film[a] | Film break | Yield (%) |
|---|---|---|---|---|---|---|---|
| 1 | 28 | 335 | 2.8 | 1 | 4 | No | < 2 |
| 2 | 14 | 200 | 1.4 | 1 | 4 | No | |
| 3 | 10.5 | 188 | 1.05 | 1 | 4 | No | |
| 4 | 7 | 57.5 | 0.7 | 1 | 2 | No | |
| 5 | 6.125 | 17 | 0.6125 | 1 | 2 | Yes | |
| 6 | 5.25 | 16.5 | 0.525 | 1 | 2 | Yes | |
| 7 | 4.375 | 13 | 0.4375 | 1 | 2 | Yes | |
| 8 | 3.5 | 11 | 0.35 | 1 | 2 | Yes | |

[a]4 = presence of thin film and fiber
2 = presence of thin film and tiny amount of fiber

## Preparation of Microparticles by Modified Laboratory Fluid Bed Spray Dryer

### Influence of Operating Conditions on the Percent Recovery of Microparticles

#### Solution Feed Rate (ml/hr)

The optimal setting for the peristaltic pump was found to be 30 ml/hr. Lower and higher solution feed rates to the peristaltic pump did not provide a maximum percent recovery. The percent recovery of microparticles was reduced from 22.57% to 17.7% and 22.57% to 7.95% with the change in feed rate from 30 to 15 ml/hr and 30 to 45 ml/hr, respectively (Table 2.6). At lower feed rates, the solution passes through the nozzle slowly, which prolongs the time for the complete drying process. Since the microparticles were collected at the top of the spray chamber, increasing the processing time would increase the chance of removing the microparticles from the collector.

At higher feed rates, the solution passes through the nozzle more quickly resulting in the formation of larger size droplets. The fluidized air has to be strong enough to quickly evaporate all the solvent, otherwise, the formed droplets will fuse together and form a thin film around the chamber's wall resulting in low recovery of microparticles. In one study it was shown that low atomized air pressure with a high feed rate can also result in reduced recovery due to the product deposition on the chamber's wall (Wan et al., 1991). Products deposition at the chamber wall was

reported to be due to semi wet particles or stickiness of the spray-dried materials (Bodmeier and Chen, 1988; Maa et al., 1998). In the study presented by Esposito et al. (2000) showed that the lowest percent recovery was achieved from microparticles prepared with the highest feed rate.

Solution feed rate also had an effect on the size of microparticles. Increasing the solution feed rate from 25 to 40 ml/hr lead to an increase in the microparticles' diameter from 2.75 to 3.33 μm (Table 2.14). Masters et al. (1985) reported that particle size of the spray-dried product depends upon feed concentration, solution viscosity, and feed rate. An increase in feed concentration, solution viscosity, and feed rate enhance the particles contact with each other and consequently increases the size of microparticles (Crosby and Marshall, 1958; Freitas and Muller, 1998; Master, 1985).

**Atomized Air Pressure (psi)**

Decreasing the atomized air pressure from 15 to 5 psi led to a reduction of microparticle percent recovery from 39.44% to 34.02% due to insufficient atomized air pressure (Wan et al., 1991). At lower atomized air pressures, the droplet of polymer solution dries more slowly compared to higher atomized air pressures, leading to the deposition of semi wet particles and the formation of a thin film around the spray chamber's wall resulting in low percent recovery of microparticles. Conversely, high air pressure also reduces the percent recovery of microparticles by

increasing the amount of particles being removed from the spray chamber. A reduction of percent recovery of microparticles from 39.44% to 34.55% was observed with an increase in atomized air pressure from 15 psi to 20 psi (Table 2.6). The optimal condition was found to be 15 psi.

The atomized air pressure was also found to affect the microparticles' size. The reduction of the microparticles' diameter from 3.335 μm to 1.323 μm was observed when the atomized air pressure was increased from 5 to 20 psi (Table 2.14) showing that an increase in atomized air pressure leads to the reduction of microparticles size. Particles size usually decreases as the atomized air pressure increases (Master, 1985).

**Drying Temperature (°C)**

Raising the temperature yielded the best percent recovery of microparticles. The outlet-inlet temperature of 60-40 °C provided the maximum percent recovery of 26.11% compared to the outlet-inlet temperature of 50-30 °C, 40-20 °C, and 30-10 °C that gave the percent recovery of microparticles of 25.31%, 22.57%, and 23.62%, respectively (Table 2.6). However, this finding was in disagreement with the study presented by Esposito et al. (2000) where microparticles percent recovery decreased with increasing drying temperature. The drying temperature was found to have an effect on the size of microparticles. The mean diameter of microparticles decreased from 4.2 μm to 2.686 μm with the increase in the inlet temperature from 40 °C to 60

°C (Table 2.14). The temperature effect on microparticles' size highly depends on the materials used (Crosby and Marshall, 1958). It was stated that temperature had a very slight effect on crystalline materials particle size for instance sodium sulfate, while film forming materials such as coffee, the diameter of spray-dried particles was considerably reduced with increasing drying temperature (Broadhead et al., 1992; Conte et al., 1994).

**Table 2.6: Influence of experimental parameters on percent recovery of microparticles prepared by modified laboratory fluid bed spray dryer**

| Factors | Level | % Recovery[a] |
|---|---|---|
| Solution Feed Rate (ml/hr) | 15 | 17.7 |
| | 25 | 10.27 |
| | 30 | 22.57 |
| | 35 | 11 |
| | 45 | 7.95 |
| Atomized Air Pressure (psi) | 5 | 34.02 |
| | 10 | 36.85 |
| | 15 | 39.44 |
| | 20 | 34.55 |
| Drying Air Temperature (°C) (inlet-outlet) | 30-10 | 23.62 |
| | 40-20 | 22.57 |
| | 50-30 | 25.31 |
| | 60-40 | 26.11 |

[a]Percentage of weight of recovered spray-dried microparticles with respect to total weight of solid used in the preparation.

**Optimization of Drug-Polymer Ratio**

Microparticles produced by modified spray drying technique using the drug-polymer ratio of 1.5:7 and 1:7.5 provided 100% entrapment efficiency of the drug with the drug being loaded at 16.30% and 10.87% of total weight of solids present, respectively. The percent encapsulation efficiency into microparticles decreased from 95% to 60% when the amount of BSA present increased from 21.74% to 27.17% (Table 2.7). During a 2-hour period when the prepared microparticles were pretreated with simulated gastric fluid, approximately 4% and 27% of drug were released out from the microparticles prepared according to the specification of formulations 9 and 10, Figure 2.4. In general, an increase in drug loading provides an increase in the amount of unencapsulated drug (Bodmeier and Chen, 1988). The drug-polymer ratio of 1.5:7 (formulation 11) was found to provide the maximum drug that could be loaded into the microparticles (16.30%) while maintaining the enteric-coated property of Eudragit® L-30 D in the microparticles. No release of BSA into the simulated gastric fluid (pH 1.23) for 2 hours was observed. The acid pretreatment was followed by immediate placing the BSA microparticles into simulated intestinal fluid (pH 7.4) where immediate release of BSA occurred (Figure 2.4), thus formulation 11 was chosen for further study.

**Table 2.7: Effect of polymer-drug ratio on percent encapsulation efficiency[a] of microparticles prepared by modified laboratory fluid bed spray dryer**

| Formulation | Polymer:Drug | Theoretical Drug Content (%) | Actual Drug Content (%) | Encapsulation Efficiency (%) |
|---|---|---|---|---|
| 9 | 6 : 2.5 | 21.85 | 16.48 | 60 |
| 10 | 6.5 : 2 | 21.85 | 20.76 | 95 |
| 11* | 7 : 1.5 | 16.30 | 16.30 | 100 |
| 12 | 7.5 : 1 | 10.81 | 10.81 | 100 |

[a]Percentage of actual drug content in spray-dried microparticles with respect to theoretical drug content.

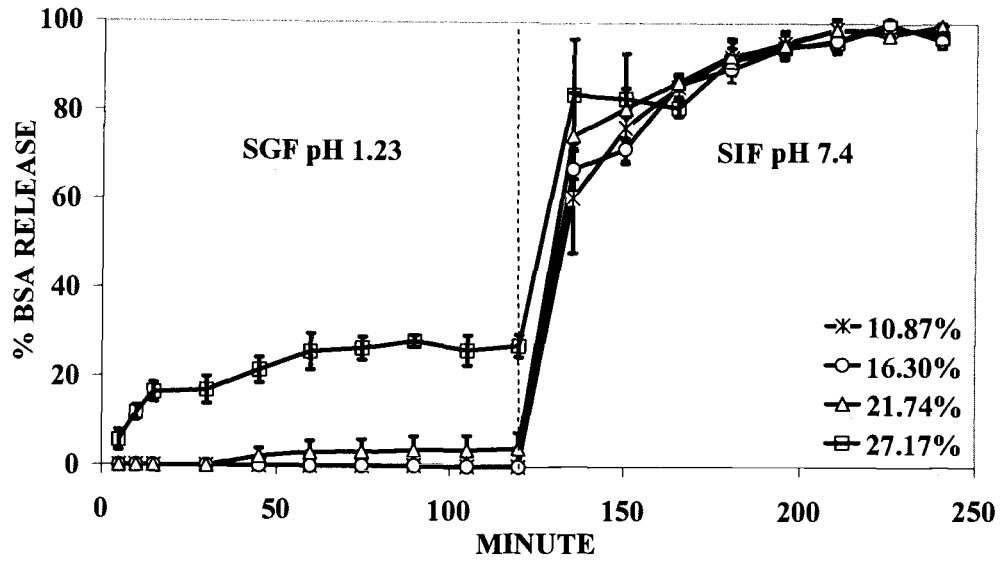



**Figure 2.4: Effect of varying percent drug load on *in vitro* release profiles of BSA from spray-dried microparticles.** Percentage of entrapped BSA release from 0.5 g Eudragit® L-30 D spray-dried microparticles was examined by dissolution test with USP apparatus II dissolution machine for 2 hours in simulated gastric fluid (SGF) pH 1.23 followed by 2 hours in simulated intestinal fluid (SIF) pH 7.4. Microparticles contained BSA content of 10.87%, 16.30%, 21.74%, and 27.17%. Mean ± SD (n=3).

**Table 2.8: Effect of varying percent drug load on *in vitro* release profiles of BSA[a] from spray-dried microparticles**

| Time(min) | Percent BSA release from microparticles with varying percent drug load | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 10.87% | SD | 16.30% | SD | 21.74% | SD | 27.17% | SD |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 | 5.62 | 2.29 |
| 10 | 0 | 0 | 0 | 0 | 0 | 0 | 11.77 | 1.62 |
| 15 | 0 | 0 | 0 | 0 | 0 | 0 | 16.43 | 2.13 |
| 30 | 0 | 0 | 0 | 0 | 0 | 0 | 16.91 | 3.06 |
| 45 | 0 | 0 | 0 | 0 | 2.09 | 1.81 | 21.52 | 2.87 |
| 60 | 0 | 0 | 0 | 0 | 3.02 | 2.62 | 25.70 | 4.06 |
| 75 | 0 | 0 | 0 | 0 | 3.19 | 2.76 | 26.43 | 2.56 |
| 90 | 0 | 0 | 0 | 0 | 3.62 | 3.14 | 28.18 | 1.30 |
| 105 | 0 | 0 | 0 | 0 | 3.72 | 3.23 | 26.14 | 3.40 |
| 120 | 0 | 0 | 0 | 0 | 4.09 | 3.55 | 27.21 | 2.48 |
| 135 | 60.48 | 12.45 | 67.04 | 4.79 | 74.58 | 9.71 | 83.75 | 12.52 |
| 150 | 76.39 | 2.55 | 71.61 | 2.55 | 80.73 | 12.34 | 82.90 | 2.38 |
| 165 | 85.57 | 2.45 | 86.10 | 2.35 | 86.66 | 1.93 | 80.86 | 1.98 |
| 180 | 92.24 | 3.69 | 89.65 | 3.00 | 92.60 | 3.91 | 91.41 | 3.00 |
| 195 | 95.46 | 2.14 | 94.45 | 2.52 | 95.06 | 3.16 | 94.45 | 2.05 |
| 210 | 98.68 | 1.52 | 96.08 | 2.89 | 98.58 | 2.47 | 95.70 | 2.00 |
| 225 | 98.46 | 1.13 | 99.97 | 0.05 | 97.33 | 0.99 | 99.50 | 0.86 |
| 240 | 98.02 | 2.07 | 96.11 | 1.70 | 99.41 | 0.57 | 96.84 | 2.28 |

[a]Percentage of entrapped BSA release from 0.5 g Eudragit® L-30 D spray-dried microparticles was examined by dissolution test with USP apparatus II dissolution machine for 2 hours in simulated gastric fluid (SGF) pH 1.23 followed by 2 hours in simulated intestinal fluid (SIF) pH 7.4. Microparticles contained BSA content of 10.87%, 16.30%, 21.74%, and 27.17%. Mean ± SD (n=3).

**Preparation of Microparticles by Solvent Evaporation Technique**

Microparticles obtained from solvent evaporation technique using Eudragit® L-30 D produced a spherical shape with their size ranging from 0.45 to 10 μm. *In vitro* dissolution drug release study showed that the percent encapsulation efficiency of Eudragit® L-30 D was about 50% with an approximately 10% initial drug release in simulated gastric fluid (Figure 2.5) which was possibly caused by the unencapsulated drug residing on the surface of particles.

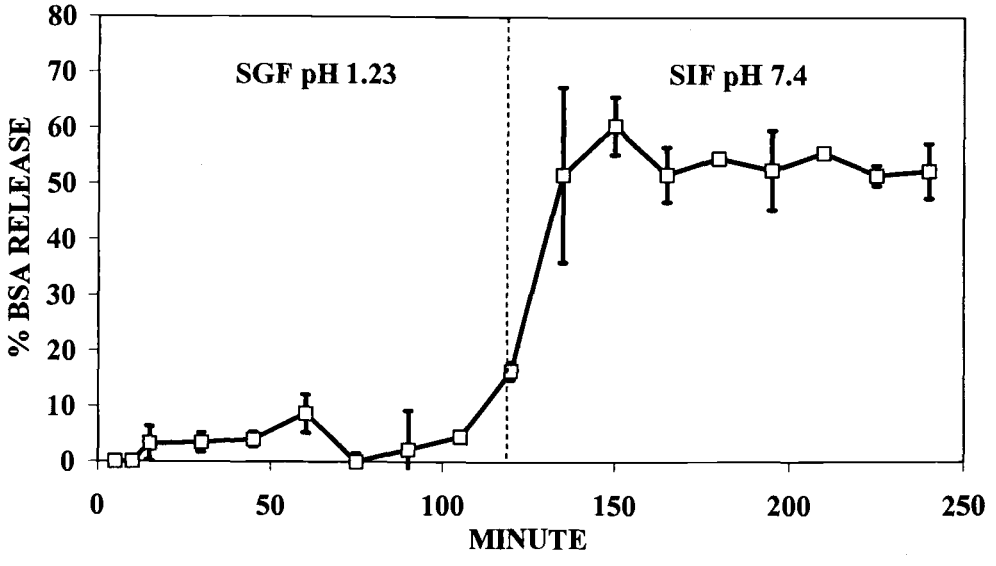



**Figure 2.5: *In vitro* release profile of BSA from microparticles produced by solvent evaporation technique.** Percentage of entrapped BSA release from 0.5 g Eudragit® L-30 D suspension microparticles was examined by dissolution test with USP apparatus III dissolution machine for 2 hours in simulated gastric fluid (SGF) pH 1.23 followed by 2 hours in simulated intestinal fluid (SIF) pH 7.4. Microparticles contained BSA content of 16.30%. Mean ± SD (n=3).

**Table 2.9: *In vitro* release profile of BSA[a] from microparticles produced by solvent evaporation technique**

| Time(min) | % BSA release | SD |
|---|---|---|
| 5 | 0 | 0 |
| 10 | 0 | 0 |
| 15 | 3.25 | 3.08 |
| 30 | 3.45 | 1.75 |
| 45 | 4.04 | 1.33 |
| 60 | 8.69 | 3.44 |
| 75 | 0.00 | 1.50 |
| 90 | 2.24 | 7.02 |
| 105 | 4.52 | 0.88 |
| 120 | 16.43 | 1.57 |
| 135 | 51.72 | 15.70 |
| 150 | 60.43 | 5.18 |
| 165 | 51.71 | 4.91 |
| 180 | 54.65 | 0.82 |
| 195 | 52.47 | 7.11 |
| 210 | 55.58 | 0.91 |
| 225 | 51.57 | 1.85 |
| 240 | 52.30 | 4.94 |

[a]Percentage of entrapped BSA release from 0.5 g Eudragit® L-30 D suspension microparticles was examined by dissolution test with USP apparatus III dissolution machine for 2 hours in simulated gastric fluid (SGF) pH 1.23 followed by 2 hours in simulated intestinal fluid (SIF) pH 7.4. Microparticles contained BSA content of 16.30%. Mean ± SD (n=3).

## *In vitro* Drugs Release from Spray-Dried Microparticles

### Evaluation of the Enteric-Coated Properties of Eudragit® L-30 D, HPMCP-55 and CAP on the Release Profile of BSA Loaded into Microparticles

Microparticles loaded with BSA prepared with Eudragit® L-30 D, HPMCP-55 or CAP gave the desired release profile. No release of BSA was observed into the simulated gastric fluid (pH 1.23) for 2 hours. After the acid pretreatment, there was an immediate release of BSA into the simulated intestinal fluid (pH 7.4) (Figure 2.6). The BSA release profile from microparticles prepared using Eudragit® L-30 D, HPMCP-55 and CAP were similar. The release pattern of plasmid DNA (pEGFP-N1) from microparticles prepared with Eudragit® L-30 D, HPMCP-55 and CAP (Figure 2.7) was similar to the BSA embedded microparticles. These results support that enteric coated microparticles could be successfully prepared with Eudragit® L-30 D, HPMCP-55 and CAP. This is similar to the study presented by Amorim and Ferreira (2001) where aprotinin protein was effectively protected from the acidic environment in the gut by microencapsulation into cellulose acetate phthalate polymer.

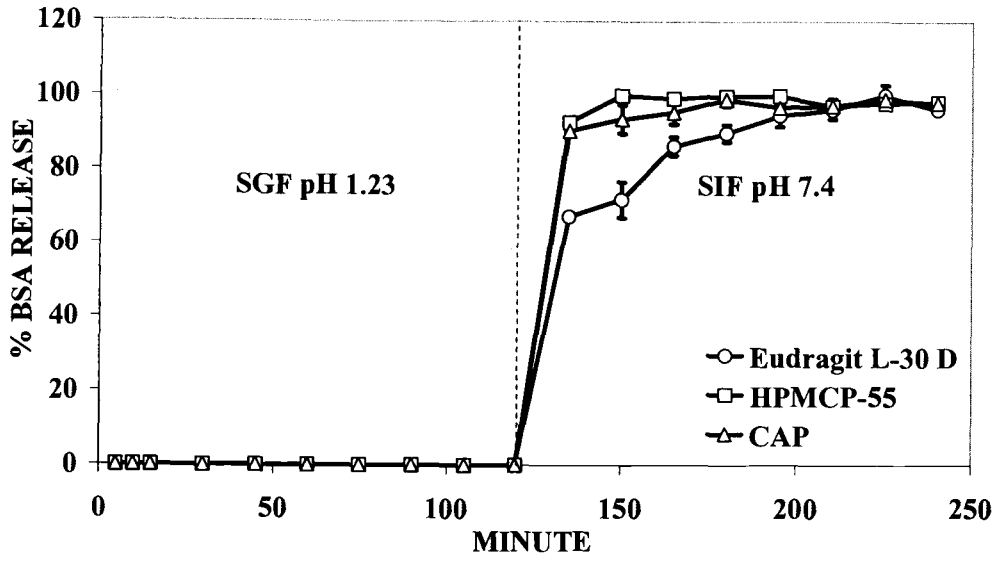



**Figure 2.6: Effect of polymer type on *in vitro* release profiles of BSA from spray-dried microparticles.** Percentage of entrapped BSA release from 0.5 g spray-dried microparticles was examined by dissolution test with USP apparatus II dissolution machine for 2 hours in simulated gastric fluid (SGF) pH 1.23 followed by 2 hours in simulated intestinal fluid (SIF) pH 7.4. Microparticles were prepared with three different types of enteric coated polymer (Eudragit L-30D, HPMCP-55, and CAP) and with actual drug loading of 16.30%. Mean ± SD (n=3).

**Table 2.10: Effect of polymer type on *in vitro* release profiles of BSA[a] from spray-dried microparticles**

| Time(min) | Percent BSA release from enteric protected microparticles | | | | | |
|---|---|---|---|---|---|---|
| | Eudragit L-30D | SD | HPMCP-55 | SD | CAP | SD |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | 0 | 0 | 0 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 | 0 | 0 | 0 |
| 45 | 0 | 0 | 0 | 0 | 0 | 0 |
| 60 | 0 | 0 | 0 | 0 | 0 | 0 |
| 75 | 0 | 0 | 0 | 0 | 0 | 0 |
| 90 | 0 | 0 | 0 | 0 | 0 | 0 |
| 105 | 0 | 0 | 0 | 0 | 0 | 0 |
| 120 | 0 | 0 | 0 | 0 | 0 | 0 |
| 135 | 67.04 | 4.79 | 92.59 | 0.05 | 90.26 | 3.90 |
| 150 | 71.61 | 2.55 | 99.91 | 0.13 | 93.43 | 3.24 |
| 165 | 86.10 | 2.35 | 99.06 | 0.60 | 95.32 | 1.79 |
| 180 | 89.65 | 3.00 | 99.69 | 0.24 | 98.75 | 1.77 |
| 195 | 94.45 | 2.52 | 99.83 | 0.24 | 96.66 | 2.05 |
| 210 | 96.08 | 2.89 | 97.05 | 0.99 | 97.10 | 1.53 |
| 225 | 99.97 | 0.05 | 97.81 | 0.83 | 98.86 | 1.61 |
| 240 | 96.11 | 1.70 | 98.16 | 0.28 | 97.97 | 1.27 |

[a]Percentage of entrapped BSA release from 0.5 g spray-dried microparticles was examined by dissolution test with USP apparatus II dissolution machine for 2 hours in simulated gastric fluid (SGF) pH 1.23 followed by 2 hours in simulated intestinal fluid (SIF) pH 7.4. Microparticles were prepared with three different types of enteric coated polymer (Eudragit L-30D, HPMCP-55, and CAP) and with actual drug loading of 16.30%. Mean ± SD (n=3).

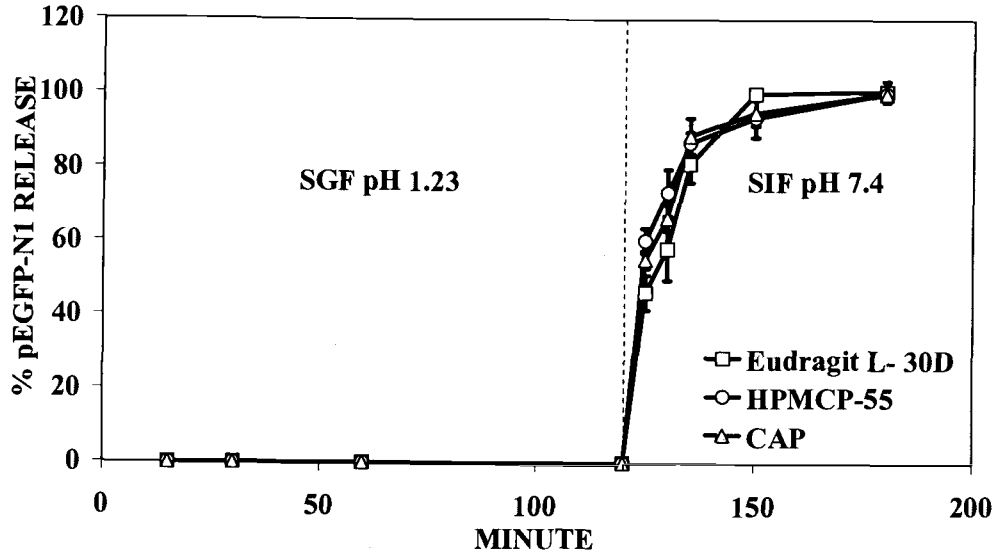



**Figure 2.7: Effect of polymer type on *in vitro* release profiles of pEGFP-N1 from spray-Dried microparticles.** Percentage of entrapped pEGFP-N1 release from 20 mg spray-dried microparticles was examined by dissolution test for 2 hours in simulated gastric fluid (SGF) pH 1.23 followed by 2 hours in simulated intestinal fluid (SIF) pH 7.4. Microparticles were prepared with three different types of enteric coated polymer (Eudragit® L-30 D, HPMCP-55, and CAP) and with actual drug loading of 16.30%. Mean ± SD (n=3).

**Table 2.11: Effect of polymer type on *in vitro* release profiles of pEGFP-N1[a] from spray-Dried microparticles**

| Time(min) | Percent pEGFP-N1 release from enteric protected microparticles | | | | | |
|---|---|---|---|---|---|---|
| | Eudragit L- 30D | SD | HPMCP-55 | SD | CAP | SD |
| 15 | 0 | 0 | 0 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 | 0 | 0 | 0 |
| 60 | 0 | 0 | 0 | 0 | 0 | 0 |
| 120 | 0 | 0 | 0 | 0 | 0 | 0 |
| 125 | 46.08 | 4.75 | 60.15 | 3.35 | 55.04 | 2.39 |
| 130 | 57.95 | 8.49 | 73.11 | 6.32 | 66.34 | 3.55 |
| 135 | 80.90 | 5.02 | 86.60 | 2.89 | 88.45 | 4.85 |
| 150 | 100 | 0 | 93.47 | 5.21 | 94.84 | 3.50 |
| 180 | 100.64 | 2.82 | 100 | 0 | 100 | 0 |

[a]Percentage of entrapped pEGFP-N1 release from 20 mg spray-dried microparticles was examined by dissolution test for 2 hours in simulated gastric fluid (SGF) pH 1.23 followed by 2 hours in simulated intestinal fluid (SIF) pH 7.4. Microparticles were prepared with three different types of enteric coated polymer (Eudragit® L-30 D, HPMCP-55, and CAP) and with actual drug loading of 16.30%. Mean ± SD (n=3).

**Analysis of Structural and Functional Integrity of Plasmid DNA (pEGFP-N1)**

Structural integrity of the plasmid DNA extracted from spray-dried microparticles was examined by agarose gel electrophoresis. The relaxed and supercoiled forms were observed in both extracted plasmid DNA and control plasmid DNA. However, extracted plasmid DNA showed a higher proportion of relaxed form compared to control plasmid DNA (Figure 2.8). This is possibly due to the denaturation of plasmid DNA during the process of microparticles' preparation. A spray dryer with the co-current manner is known as a suitable method for the

preparation of heat sensitive materials because of the cooling effect caused by moisture evaporation. As soon as the liquid droplet is in contact with the air stream, the moisture begins to evaporate and the polymer shell starts to form at the surface of the droplets, which limits solvent evaporation. At this stage, the temperature increases gradually and spray-dried products become sensitive to heat. As a general rule, the loss in activity of thermo sensitive materials depends strongly on temperature and the time of temperature exposure as well as the nature of heat sensitive materials used. Usually, the time materials are exposed to the elevated temperature is about 5 to 30 seconds (Broadhead et al., 1992).

Roa and Mathur, (1987) observed the lack of α-lactoglobulin in infant milk powder when the spray drying temperature was increased from 95 °C to 110 °C, which indicated the occurrence of protein denaturation (Broadhead et al., 1992). Galyean and Cotterill reported that the denaturation of egg white globulin during spray the drying process was the result of shear forces during atomization.

The functional integrity was determined by transfection of extracted plasmid DNA into human adenocarcinoma cell line, Caco-2 cells. The observation of a green fluorescence region in culture cells caused by gene expression of the plasmid DNA in Caco-2 cells revealed that extracted plasmid DNA was capable of transfecting the cells. These results suggest that the plasmid DNA embedded into spray-dried microparticles still retained their structural and functional integrity.

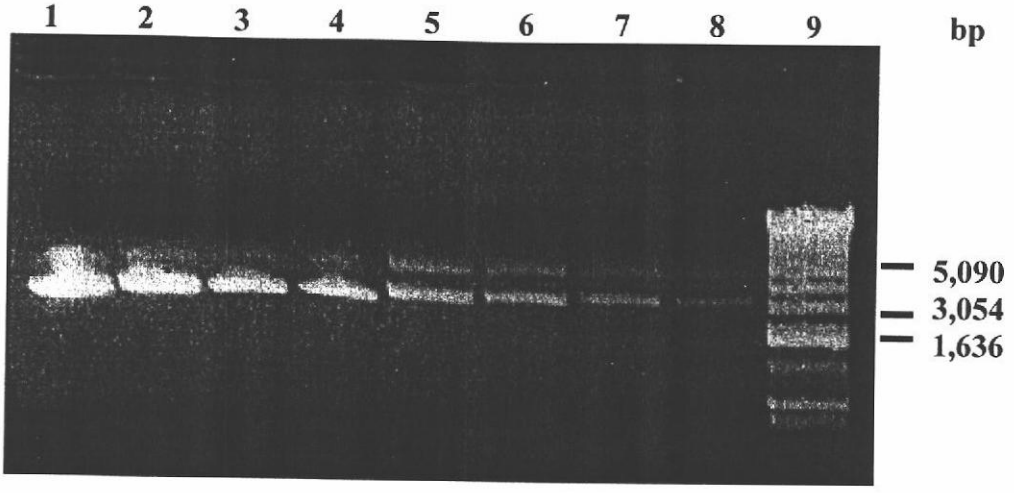



**Figure 2.8: Agarose gel electrophoresis analysis of pEGFP-N1 extracted from Eudragit® L-30 D spray-dried microparticles.** Lane 1-lane 4 is 0.5, 0.25, 0.125 and 0.1 μg control pEGFP-N1, respectively. Lane 5-lane 8 is extracted pEGFP-N1 in the amount of 0.125, 0.1, 0.05, and 0.025 μg, respectively. Lane 9 is DNA ladder.

**Influence of pH Adjusting Agents on the Release Profile and Morphology of Eudragit® L-30 D Microparticles**

The release of BSA from spray-dried microparticles using sodium hydroxide to adjust pH was faster than spray-dried microparticles using ammonium hydroxide for pH adjustment (Figure 2.9). During the spray drying process, ammonium evaporated away while sodium was left behind with the Eudragit® L-30 D molecules resulting in increased the dissolution rate. Eudragit® L-30 D is an acid molecule. If ammonium evaporates during spray drying Eudragit® L-30 D remains in the unionized state and will dissolve more slowly as it must ionize first to dissolve. Having sodium present with Eudragit® L-30 D keep the polymer in the ionized form and in this form it will dissolve faster releasing BSA sooner. As a general rule, sodium salts of compounds dissolve more rapidly compared to the unionized form.

$$\text{Eudragit® L-30 D} + NH_4OH + H_2O \rightarrow \text{Eudragit® L-30 D} + NH_3 + H_2O$$

$$\text{Eudragit® L-30 D} + NaOH + H_2O \rightarrow \text{Eudragit® L-30 D-Na} + H_2O$$

Sodium hydroxide was observed to produce smooth surface microparticles while rough surface microparticles were obtained using ammonium hydroxide (Figure 2.10).

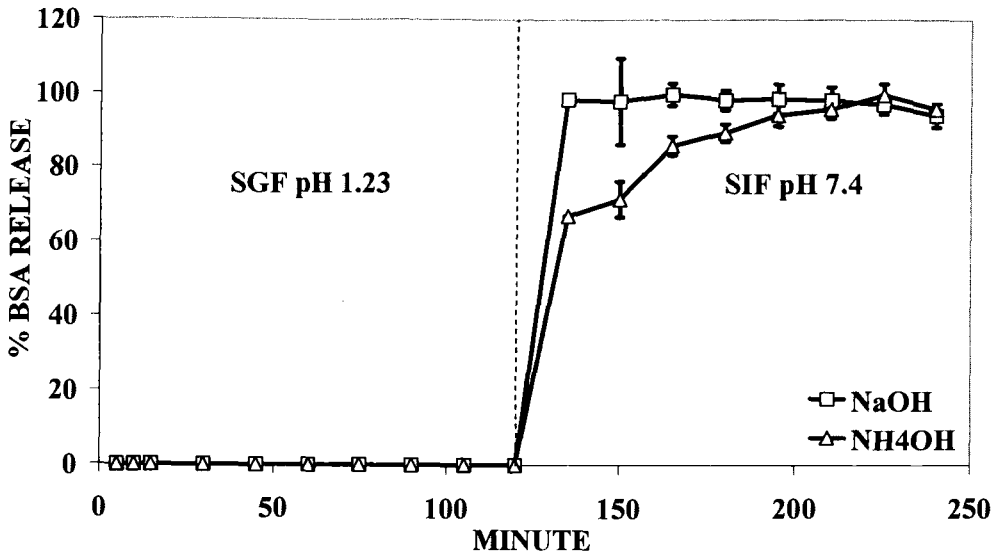


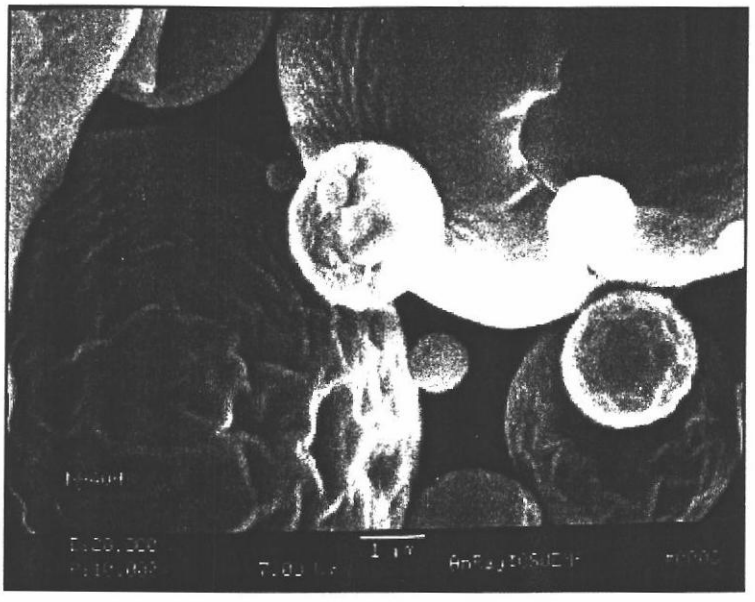

**Figure 2.9: Effect of pH adjusting agents on *in vitro* release profiles of BSA from spray-dried microparticles.** Percentage of entrapped BSA release from 0.5 g Eudragit® L-30 D spray-dried microparticles was examined by dissolution test with USP apparatus II dissolution machine for 2 hours in simulated gastric fluid (SGF) pH 1.23 followed by 2 hours in simulated intestinal fluid (SIF) pH 7.4. Microparticles were prepared with two different types of pH adjusting agents, sodium hydroxide (NaOH) or ammonium hydroxide ($NH_4OH$), and with actual drug loading of 16.30%. Mean ± SD (n=3).

**Table 2.12: Effect of pH adjusting agents on *in vitro* release profiles of BSA[a] from spray-dried microparticles**

| Time (min) | % BSA release from Eudragit L-30D-NaOH | SD | % BSA release from Eudragit L-30D-NH4OH | SD |
|---|---|---|---|---|
| 5 | 0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 |
| 15 | 0 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 | 0 |
| 45 | 0 | 0 | 0 | 0 |
| 60 | 0 | 0 | 0 | 0 |
| 75 | 0 | 0 | 0 | 0 |
| 90 | 0 | 0 | 0 | 0 |
| 105 | 0 | 0 | 0 | 0 |
| 120 | 0 | 0 | 0 | 0 |
| 135 | 98.44 | 11.69 | 67.04 | 4.79 |
| 150 | 98.01 | 3.00 | 71.61 | 2.55 |
| 165 | 100.00 | 2.70 | 86.10 | 2.35 |
| 180 | 98.55 | 3.97 | 89.65 | 3.00 |
| 195 | 98.81 | 3.56 | 94.45 | 2.52 |
| 210 | 98.45 | 2.53 | 96.08 | 2.89 |
| 225 | 97.32 | 3.19 | 99.97 | 0.05 |
| 240 | 94.38 | 2.89 | 96.11 | 1.70 |

[a]Percentage of entrapped BSA release from 0.5 g Eudragit® L-30 D spray-dried microparticles was examined by dissolution test with USP apparatus II dissolution machine for 2 hours in simulated gastric fluid (SGF) pH 1.23 followed by 2 hours in simulated intestinal fluid (SIF) pH 7.4. Microparticles were prepared with two different types of pH adjusting agents, sodium hydroxide (NaOH) or ammonium hydroxide ($NH_4OH$), and with actual drug loading of 16.30%. Mean ± SD (n=3).

A



B

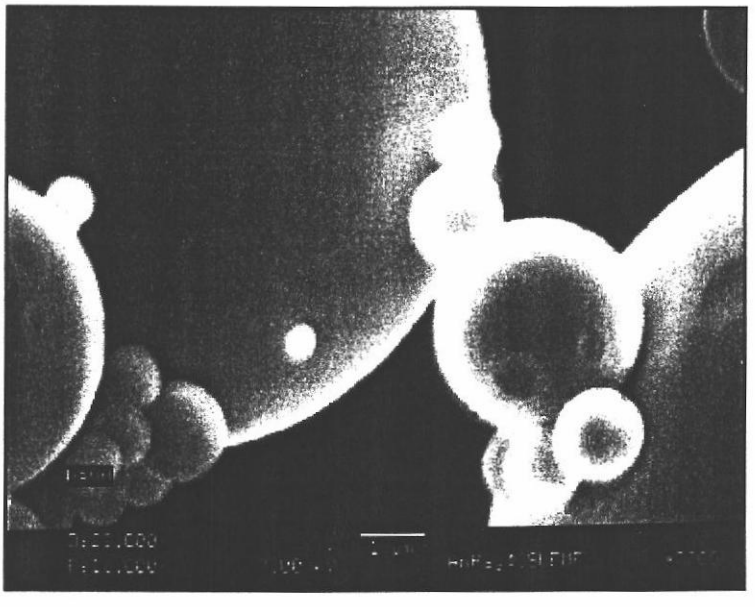


**Figure 2.10: Effect of pH adjusting agents on morphology of Eudragit® L-30 D microparticles produced by spray drying.** Scanning electron micrograph of microparticles containing bovine serum albumin prepared with ammonium hydroxide ($NH_4OH$) (Panel A) and with sodium hydroxide (NaOH) (Panel B).

## The Swelling Characteristics of Eudragit® L-30 D Microparticles

Eudragit® L-30 D is a pH-dependent polymer, which will not dissolve in acidic conditions, pH < 5.5. However, in aqueous solution, Eudragit® L-30 D tends to swell resulting in a change in microparticle size. The mean diameter of spray-dried microparticles after suspending in simulated gastric fluid (pH 1.23) increased with time and reached a plateau at about 90 minutes with the approximate diameter of 4.685 μm. This was an 80.89 % increase in the diameter of the original dried microparticles, 2.59 μm (Figure 2.11).

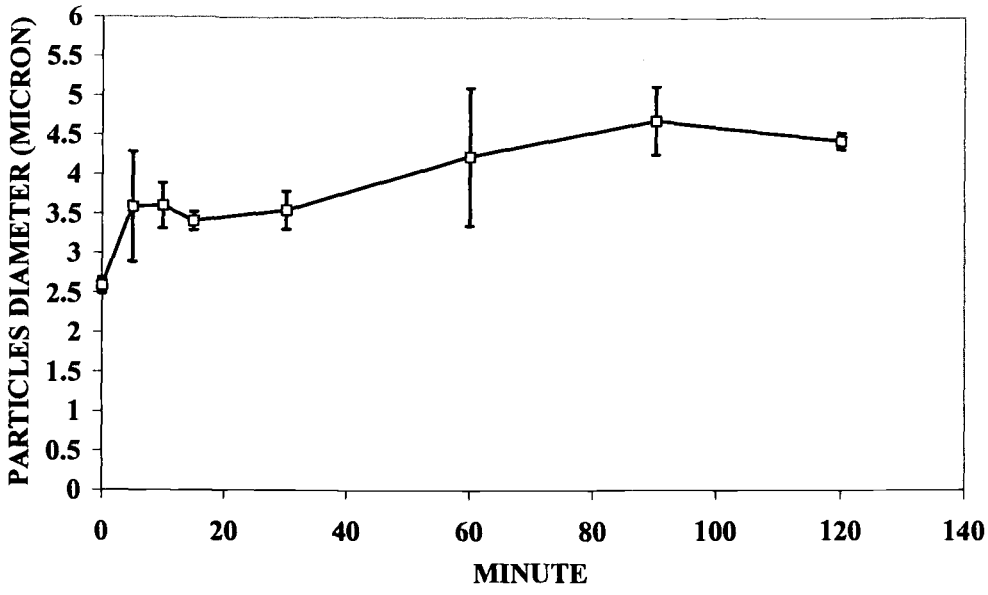



**Figure 2.11: The swelling characteristics of Eudragit® L-30 D spray-dried microparticles.** Degree of swelling of Eudragit® L-30 D spray-dried microparticles were determined by examining the change in the diameter of microparticles suspended in simulated gastric fluid pH 1.23 during 2 hours period. Mean ± SD (n=1000).

**Table 2.13: The swelling characteristics[a] of Eudragit® L-30 D spray-dried microparticles**

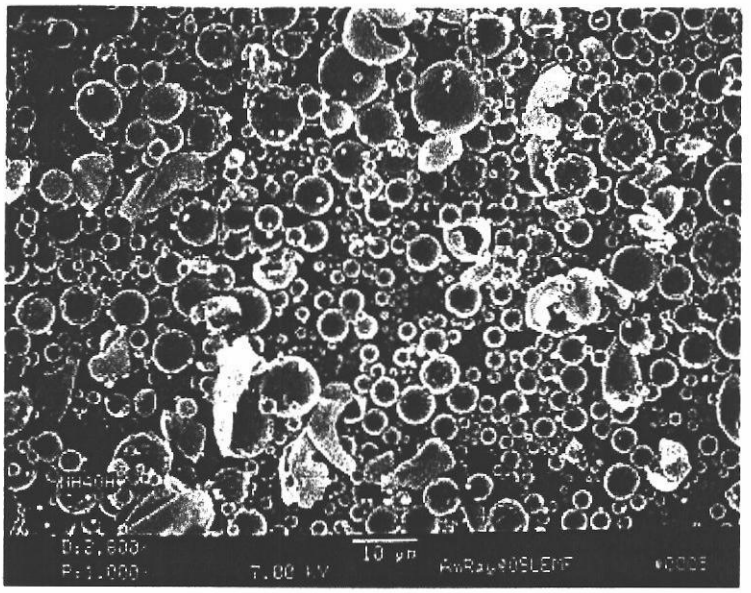
| Time | Diameter (μm) | SD |
|---|---|---|
| 0 | 2.59 | 0.10 |
| 5 | 3.59 | 0.70 |
| 10 | 3.60 | 0.29 |
| 15 | 3.41 | 0.12 |
| 30 | 3.54 | 0.24 |
| 60 | 4.23 | 0.88 |
| 90 | 4.69 | 0.43 |
| 120 | 4.43 | 0.11 |

[a]Degree of swelling of Eudragit® L-30 D spray-dried microparticles were determined by examining the change in the diameter of microparticles suspended in simulated gastric fluid pH 1.23 during 2 hours period. Mean ± SD (n=1000).

## Microparticle Morphology Analysis

The modified spray drying technique produced spherical microparticles with their size ranging from 0.18 to 20 μm (Figure 2.12A and 2.12B). The size and size distribution revealed that Eudragit® L-30 D microparticles possessed a log-normal size distribution or skewed distribution as shown in Table 2.14.

A



B

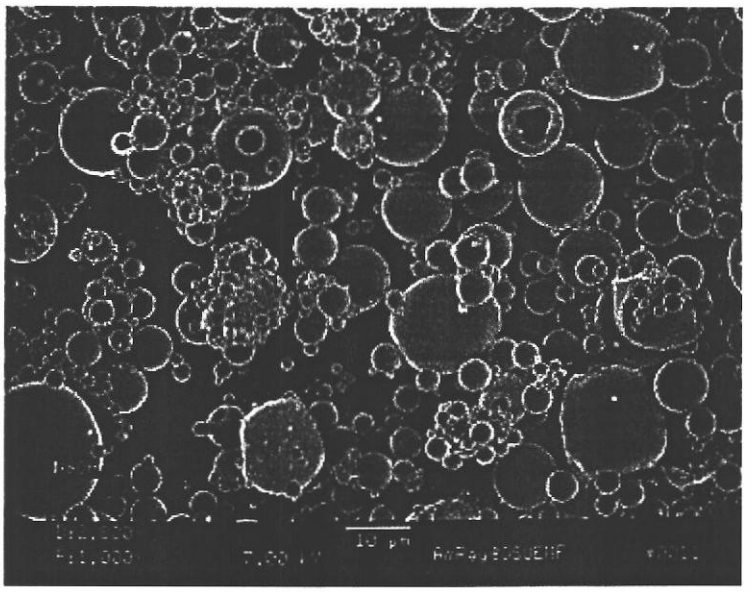


**Figure 2.12: Scanning electron micrograph (SEM) of spray-dried microparticles.** Panel A and Panel B show SEM of BSA loaded Eudragit® L-30 D spray-dried microparticles produced using ammonium hydroxide and sodium hydroxide as pH adjusting agents, respectively.

**Table 2.14: Influence of operation conditions on dimensional characteristics and percent recovery of Eudragit® L-30 D microparticles**

| Operating Conditions | Mean Diameter (μm) | SE | Skewness[b] | Kurtosis[c] | % Recovery [a] |
|---|---|---|---|---|---|
| Solution Feed Rate (ml/hr) | | | | | |
| 25 | 2.75 | 0.046 | 2.133 | 7.555 | 10.27 |
| 30 | 2.49 | 0.123 | 1.548 | 2.584 | 22.57 |
| 35 | 3.57 | 0.090 | 1.481 | 2.669 | 11.0 |
| 40 | 3.33 | 0.106 | 1.805 | 4.478 | 7.95 |
| Atomized Air Pressure (psi) | | | | | |
| 5 | 3.335 | 0.154 | 1.354 | 1.748 | 34.02 |
| 10 | 3.064 | 0.167 | 1.941 | 5.321 | 36.85 |
| 15 | 2.494 | 0.123 | 1.548 | 2.584 | 39.44 |
| 20 | 1.323 | 0.144 | 5.436 | 35.892 | 34.55 |
| Inlet-Outlet Air Temperature (°C) | | | | | |
| 30-10 | 3.635 | 0.149 | 1.421 | 2.015 | 23.62 |
| 40-30 | 4.200 | 0.203 | 1.114 | 1.122 | 22.57 |
| 50-30 | 2.742 | 0.130 | 3.570 | 18.126 | 25.31 |
| 60-40 | 2.686 | 0.179 | 3.779 | 18.442 | 26.11 |

[a]Percentage of weight of recovered spray-dried microparticles with respect to total weight of solid used in the preparation
[b]Skewness value > 0 indicates positive skewness and skewness value < 0 indicates negative skewness.
[c]Kurtosis value = 3 indicates bell shape distribution, kurtosis value > 3 indicates small tail, and kurtosis value < 3 indicates large tail.

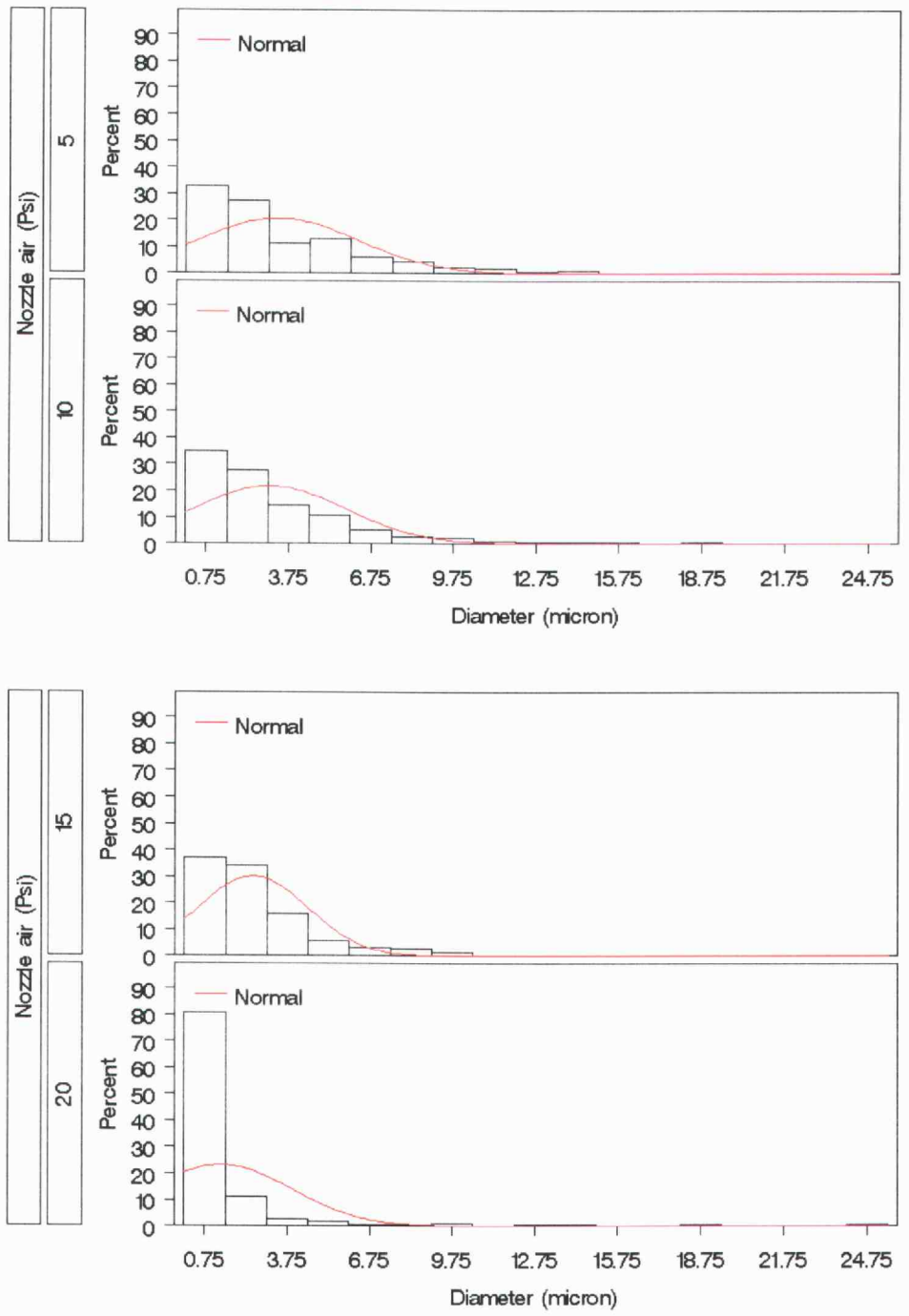



**Figure 2.13: Influence of nozzle air pressure variation on dimensional distribution of Eudragit® L-30 D spray-dried microparticles.** Particles size distribution of Eudragit® L-30 D spray-dried microparticles obtained from nozzle air of 5, 10, 15, and 20 psi showing skewed size distribution.

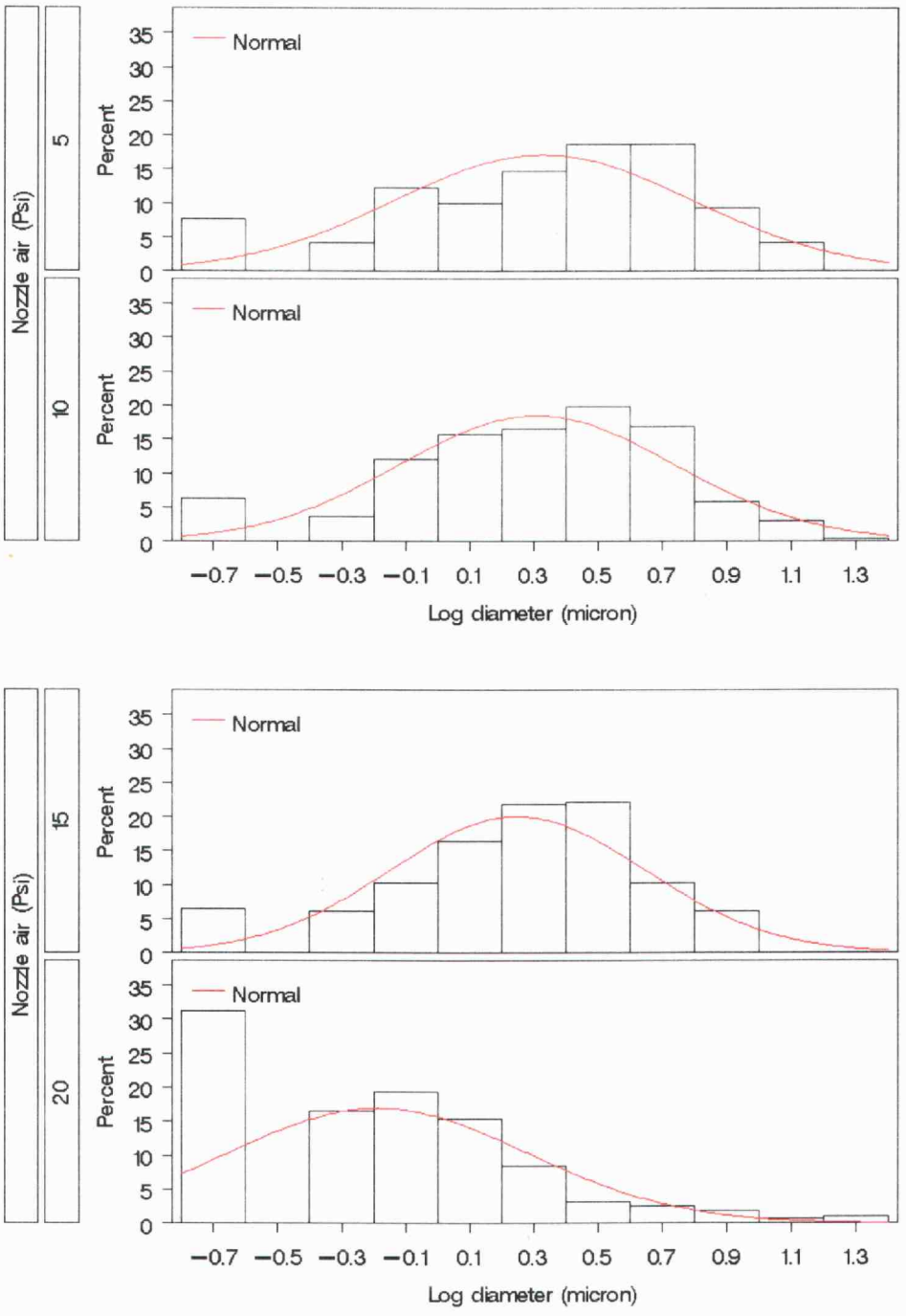



**Figure 2.14: Influence of nozzle air pressure variation on dimensional distribution of Eudragit® L-30 D spray-dried microparticles.** Particles size distribution of Eudragit® L-30 D spray-dried microparticles obtained from nozzle air of 5, 10, 15, and 20 psi showing log-normal size distribution.

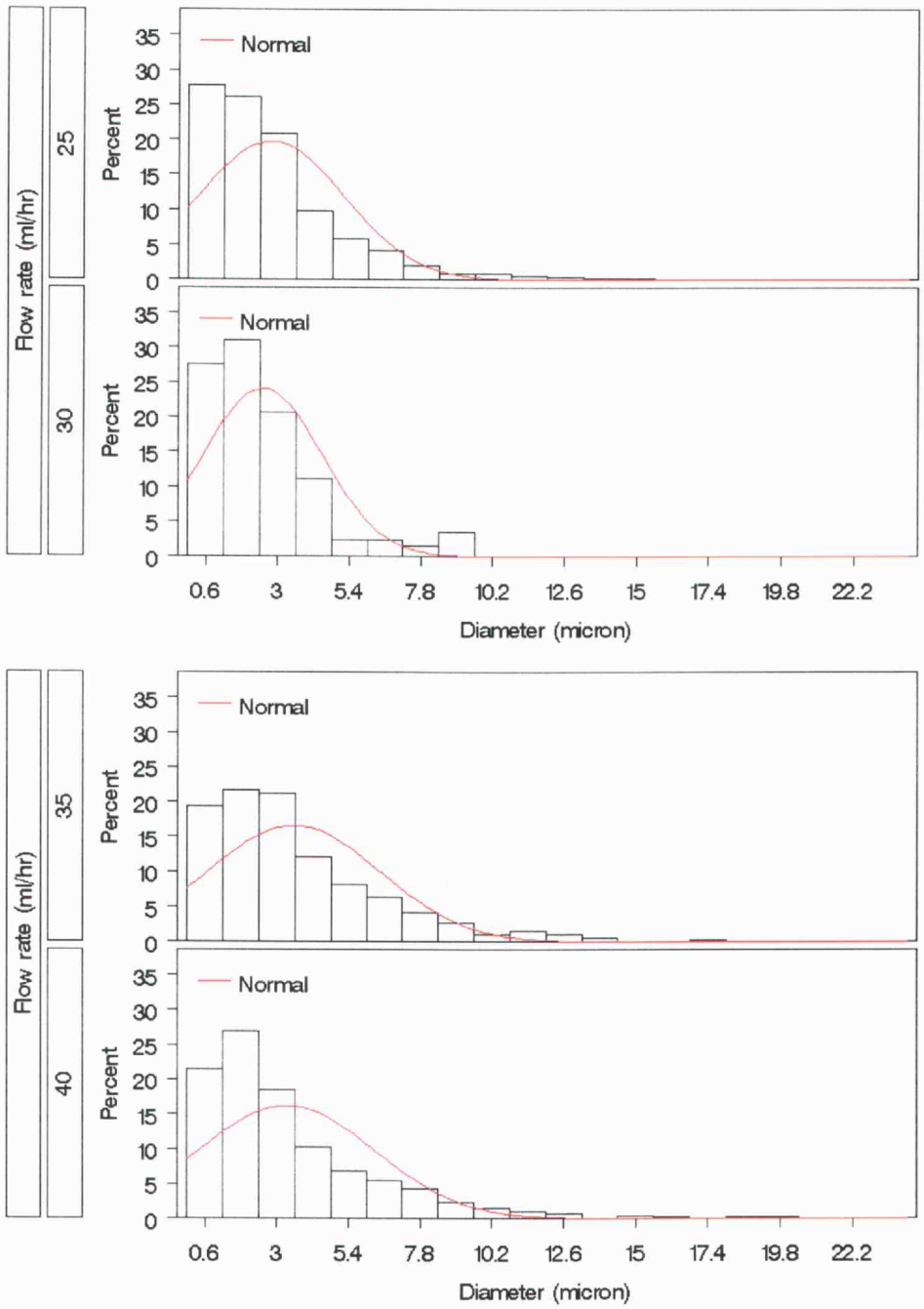



**Figure 2.15: Influence of flow rate variation on dimensional distribution of Eudragit® L-30 D spray-dried microparticles.** Particles size distribution of Eudragit® L-30 D spray-dried microparticles obtained from flow rate of 20, 30, 35, and 40 ml/hr showing skewed size distribution.

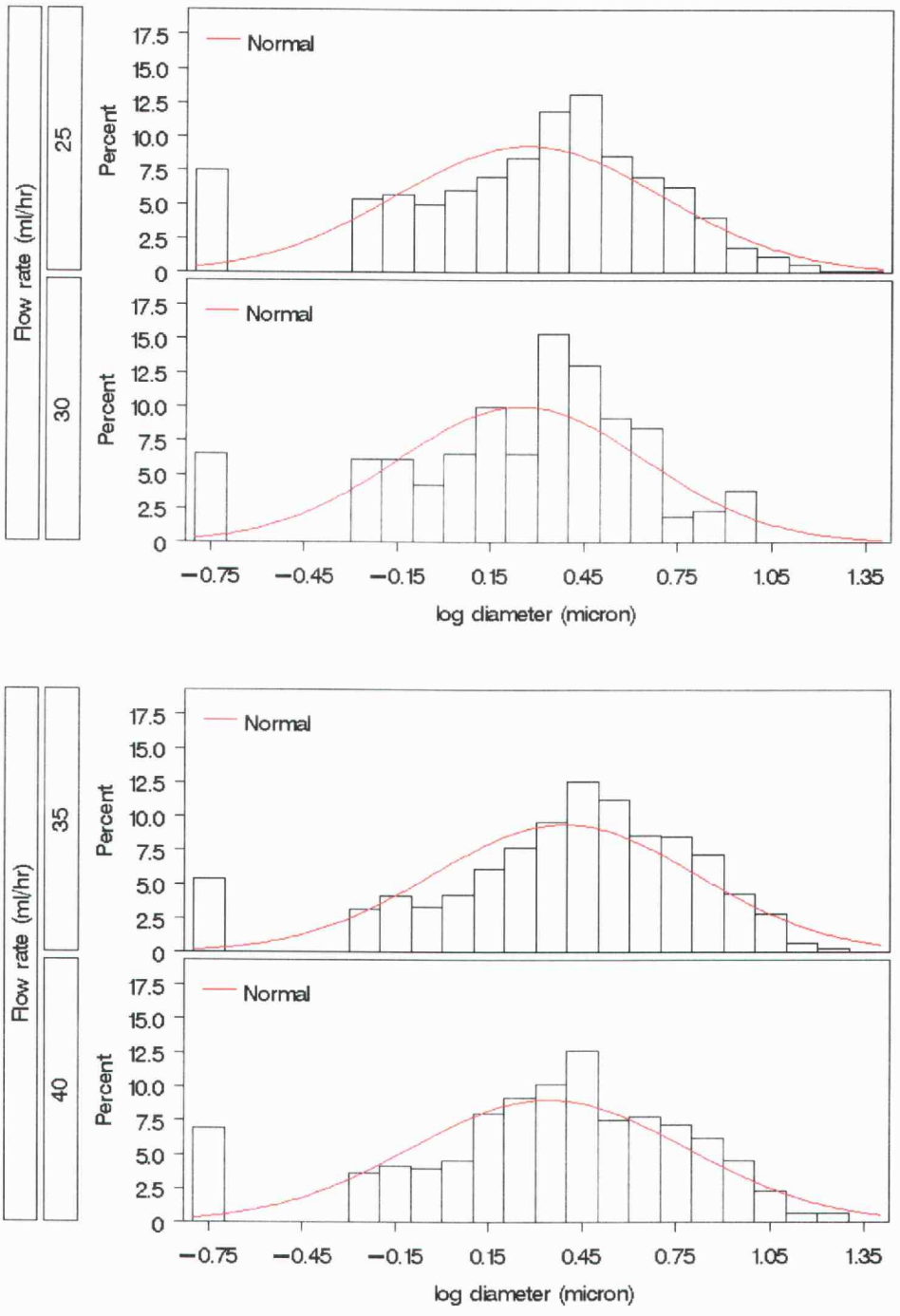



**Figure 2.16: Influence of flow rate variation on dimensional distribution of Eudragit® L-30 D spray-dried microparticles.** Particles size distribution of Eudragit® L-30 D spray-dried microparticles obtained from flow rate of 20, 30, 35, and 40 ml/hr showing log-normal size distribution.

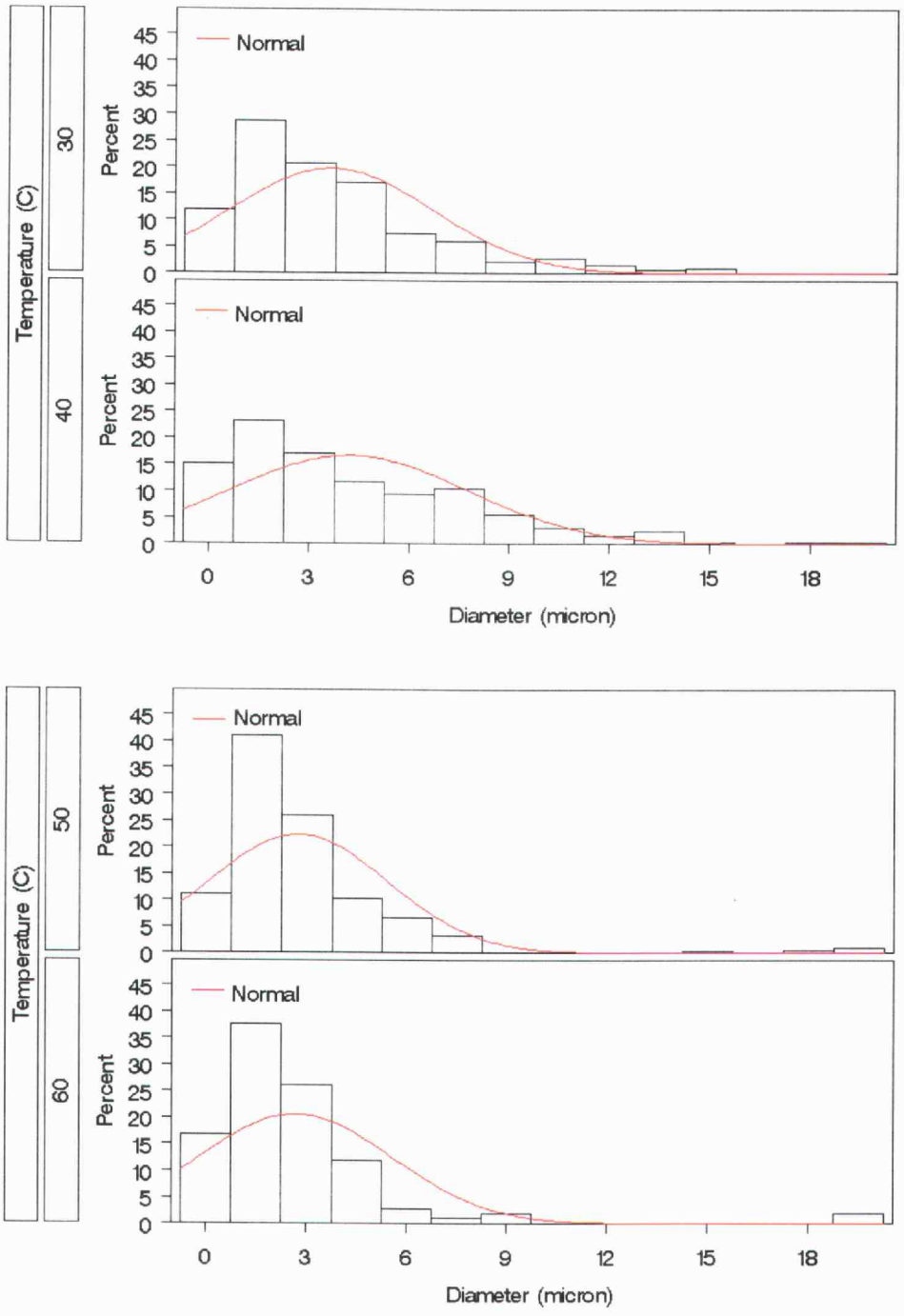



**Figure 2.17: Influence of temperature variation on dimensional distribution of Eudragit® L-30 D spray-dried microparticles.** Particles size distribution of Eudragit® L-30 D spray-dried microparticles obtained from inlet temperature of 30, 40, 50, and 60 ˚C showing skewed size distribution.

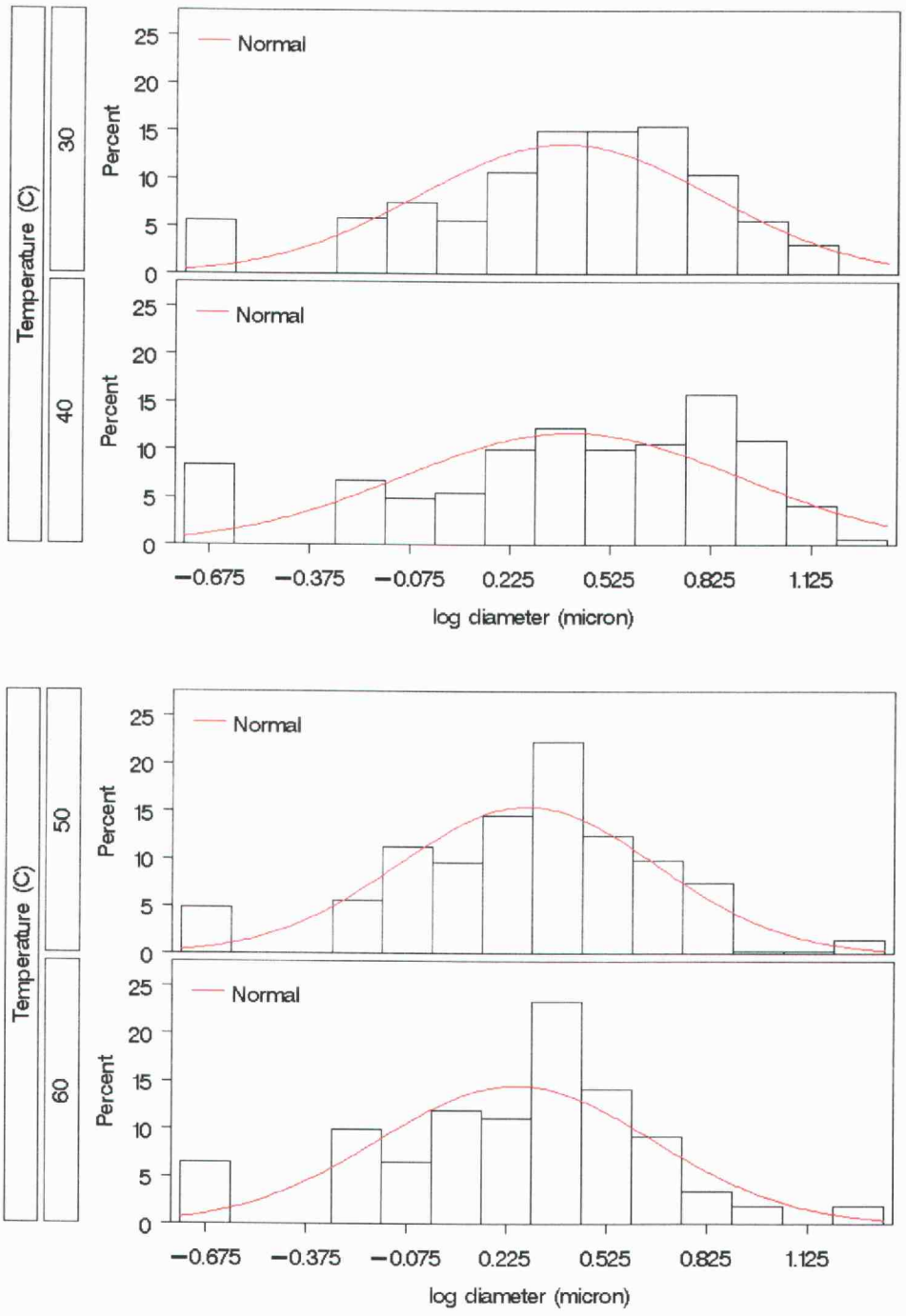



**Figure 2.18: Influence of temperature variation on dimensional distribution of Eudragit® L-30 D spray-dried microparticles.** Particles size distribution of Eudragit® L-30 D spray-dried microparticles obtained from inlet temperature of 30, 40, 50, and 60 °C showing log-normal size distribution.

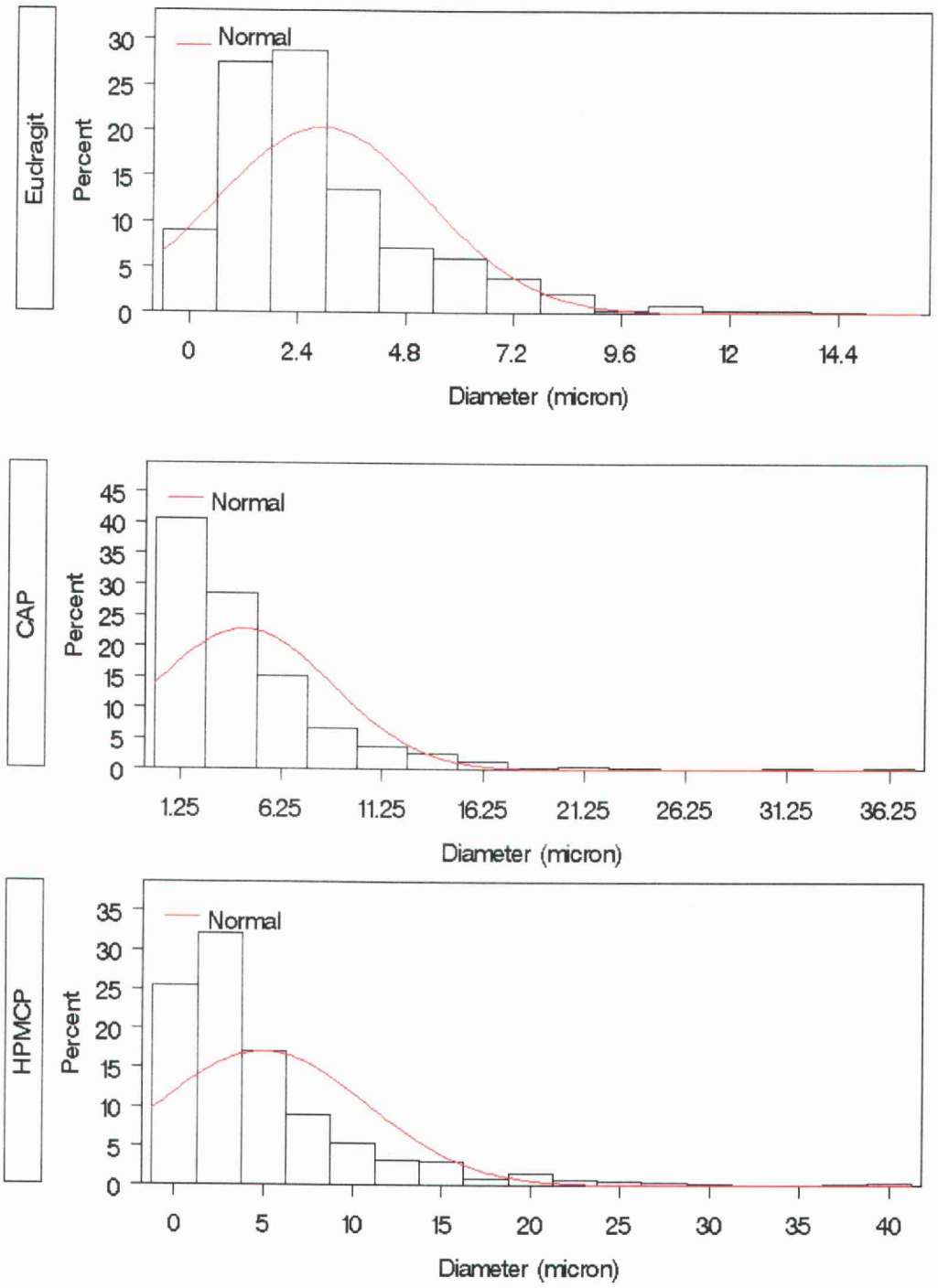



**Figure 2.19: Influence of polymer type on dimensional distribution of spray-dried microparticles.** Particles size distribution of spray-dried microparticles obtained from Eudragit® L-30 D, CAP, and HPMC polymer showing skewed size distribution.

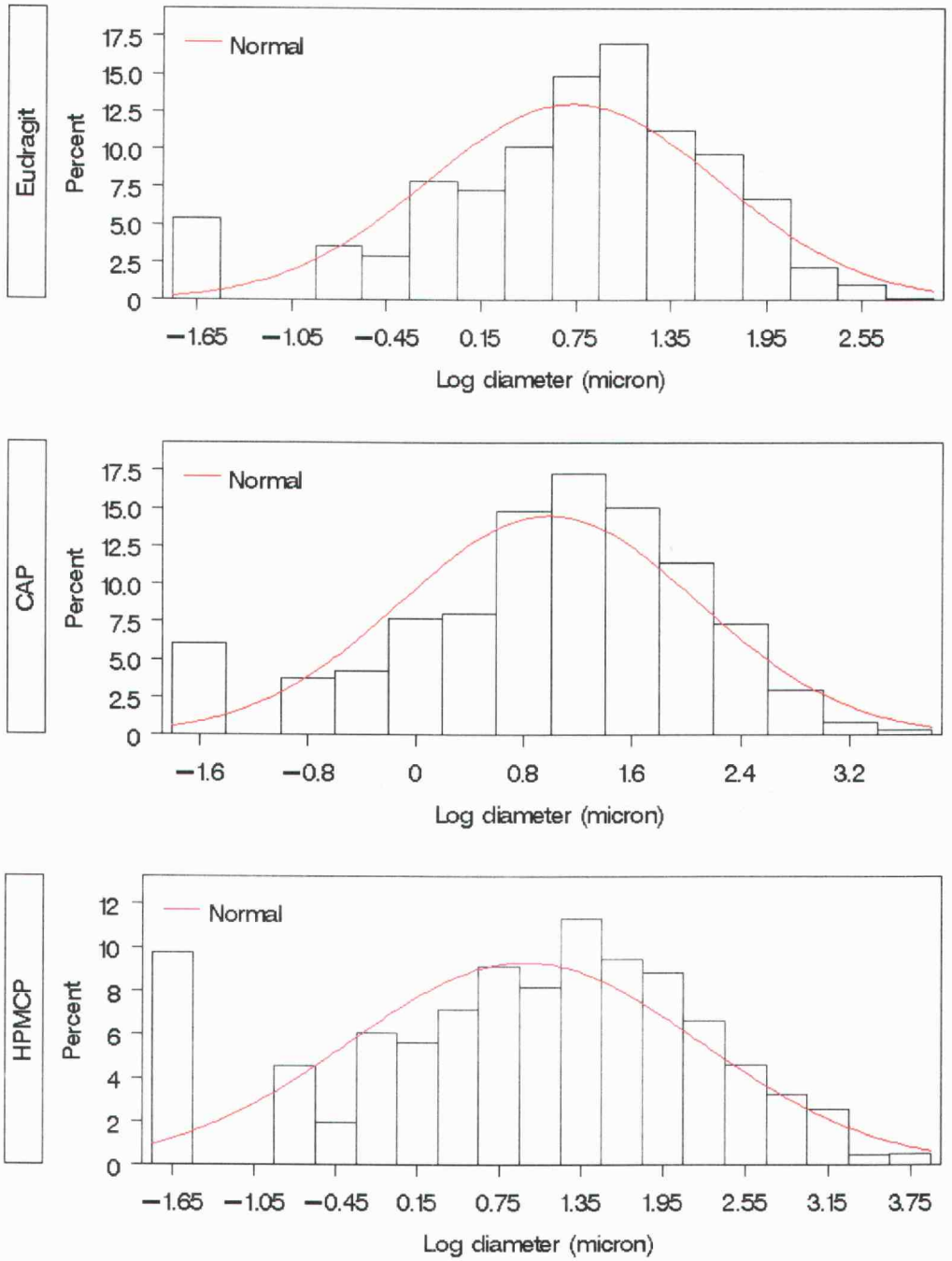



**Figure 2.20: Influence of polymer type on dimensional distribution of spray-dried microparticles.** Particles size distribution of spray-dried microparticles obtained from Eudragit® L-30 D, CAP, and HPMC polymer showing log-normal size distribution.

**The Effect of Storage Conditions on Structural Integrity of pEGFP-N1 in Microparticles**

pEGFP-N1 embedded into spray-dried microparticles prepared with Eudragit® L-30 D-$NH_4OH$, HPMCP-55, and CAP lost their integrity at room temperature (Figure 2.22) while pEGFP-N1 in microparticles stayed intact when stored at 4 °C (Figure 2.21) and –20 °C over a six month period (Table 2.15). pEGFP-N1 embedded into spray-dried microparticles prepared with Eudragit® L-30 D-NaOH stayed intact at all temperature levels, RT, 4 °C, -20 °C when stored for six months (Figure 2.21 and 2.22 and Table 2.15). Light was found to not have an effect on the integrity of pEGFP-N1 embedded in microparticles formed by any of polymers investigated, Eudragit® L-30 D, HPMCP-55, and CAP (Figure 2.23 and Table 2.15).

**Table 2.15: Effect of storage conditions on the integrity of pEGFP-N1[a] over time period**

| Polymers | Temperature[b] | RT | | | | 4 °C | | | | -20 °C | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Light[c] | L | | D | | L | | D | | L | | D | |
| | Time[d] (month) | 0 | 6 | 0 | 6 | 0 | 6 | 0 | 6 | 0 | 6 | 0 | 6 |
| Eudragit L-30D ($NH_4OH$) | | X | - | X | - | X | X | X | X | X | X | X | X |
| Eudragit L-30D (NaOH) | | X | X | X | X | X | X | X | X | X | X | X | X |
| HPMCP-55 | | X | - | X | - | X | X | X | X | X | X | X | X |
| CAP | | X | - | X | - | X | X | X | X | X | X | X | X |

[a]X represents intact of pEGFP-N1 in spray-dried microparticles.
[b]RT represents room temperature, 4 °C and -20 °C represent the temperature of 4 and -20 degree Celsius, respectively.
[c]0 and 6 represent storage time of 0 (right after preparation) and 5 months, respectively.

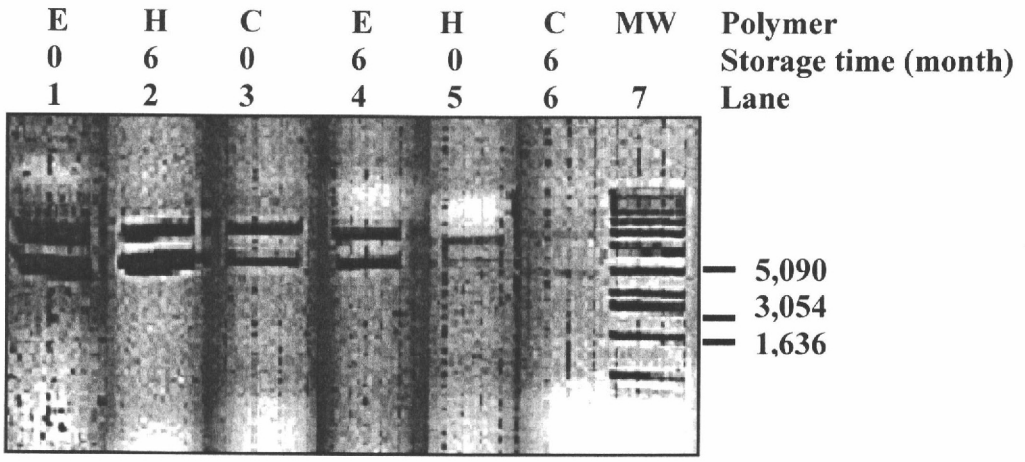



**Figure 2.21: Agarose gel electrophoresis shows effect of storage time on the integrity of pEGFP-N1.** Lane 1, 3, and 5 are pEGFP-N1 extracted from Eudragit® L-30 D (E), HPMCP-55 (H), and CAP (C) spray-dried microparticles analyzed right after preparation. Lane 2, 4, and 6 are pEGFP-N1 extracted from Eudragit® L-30 D, HPMCP-55, and CAP spray-dried microparticles analyzed 6 months post preparation. Lane 7 is DNA ladder. Microparticles were stored at 4 °C under dark condition.

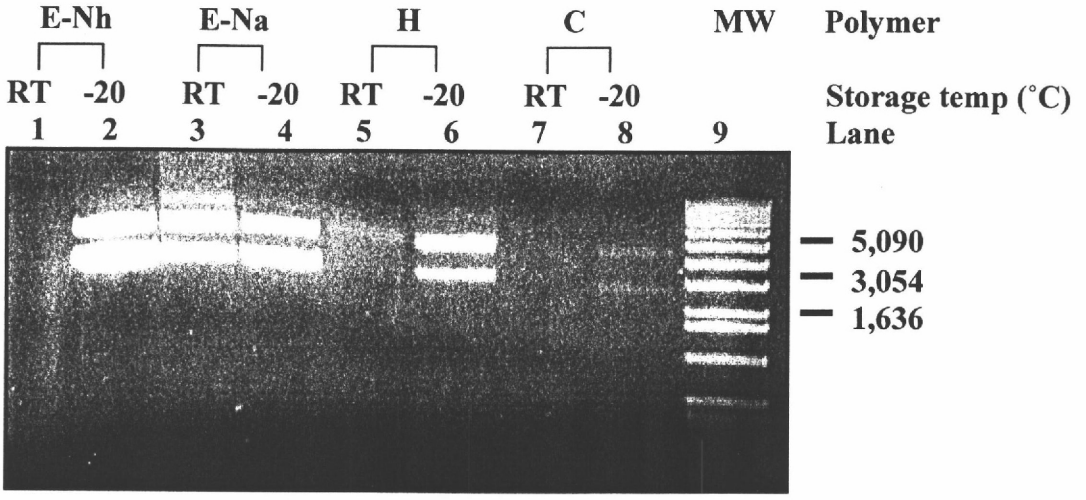



**Figure 2.22: Agarose gel electrophoresis shows effect of storage temperature on the integrity of pEGFP-N1.** Lane 1, 3, 5, 7 are pEGFP-N1 extracted from Eudragit® L-30 D-NH4OH[a] (E-Nh), Eudragit® L-30 D-NaOH[b] (E-Na), HPMCP-55 (H), and CAP (C) stored at room temperature under dark condition, respectively. Lane 2, 4, 6, 8 are pEGFP-N1 extracted from Eudragit® L-30 D-NH4OH[a], Eudragit® L-30 D-NaOH[b], HPMCP-55, and CAP stored at -20 °C under dark condition, respectively. Lane 9 is DNA ladder.

[a]Eudragit® L-30 D spray-dried microparticles using $NH_4OH$ adjusted pH.
[b]Eudragit® L-30 D spray-dried microparticles using NaOH adjusted pH.

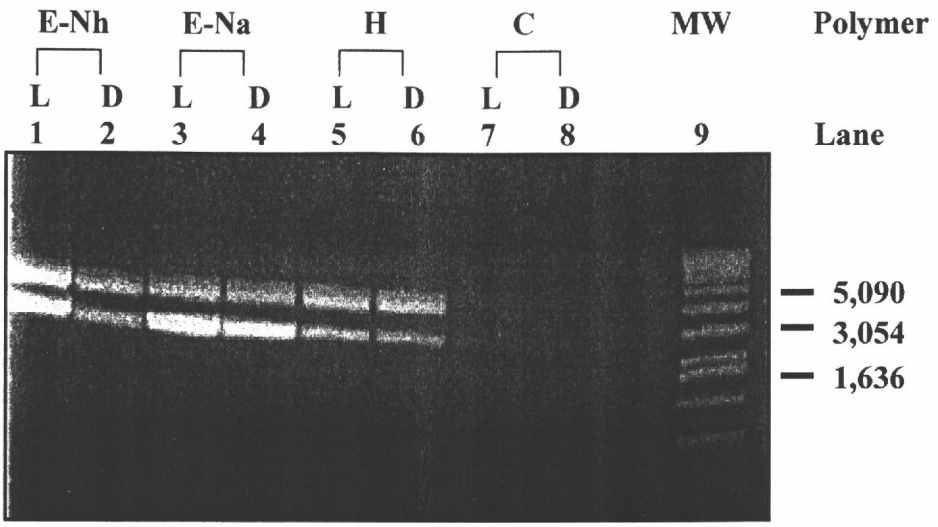



**Figure 2.23: Agarose gel electrophoresis shows effect of light on the integrity of pEGFP-N1.** Lane 1, 3, 5, 7 are pEGFP-N1 extracted from Eudragit® L-30 D-NH4OH[a] (E-Nh), Eudragit® L-30 D-NaOH[b] (E-Na), HPMCP-55 (H), and CAP (C) stored at 4 °C under light (L) condition, respectively. Lane 2, 4, 6, 8 are pEGFP-N1 extracted from Eudragit® L-30 D-NH4OH[a], Eudragit® L-30 D-NaOH[b], HPMCP-55, and CAP stored at 4 °C under dark (D) condition, respectively. Lane 9 is DNA ladder.

[a]Eudragit® L-30 D spray-dried microparticles using $NH_4OH$ adjusted pH.
[b]Eudragit® L-30 D spray-dried microparticles using NaOH adjusted pH.

## *In vitro* Uptake of Microparticles by Caco-2 cells

Gene expression was not observed in Caco-2 cells treated with either spray-dried microparticles or a suspension of microparticles in all pH ranges tested (pH 5.3 and 7.4). In general, the mechanism for microparticles uptake into cells is particle size dependent (Carr et al., 1996; Desai et al., 1997). Small particle sizes have a greater ability to cross the mucosal barrier compared to larger particle sizes (Desai et al., 1997). Although it is not clear what the size cut-off for the uptake of particles in cells is (Carr et al., 1996), spray-dried microparticles with the size range from 0.18 to 20 μm could be too large for the cells to take up. The study by Desai et al. (1997) demonstrated that the efficiency of Caco-2 cells uptake of biodegradable microparticles of 0.1 μm, 1 μm, and 10 μm at 100 μg/ml was 41%, 15%, and 6%, respectively. Carr et al. (1996) reported that the cut-off size for the uptake of particles by small intestine epithelial tissues is 10 μm. Even though smaller particle sizes in the range of 0.45 to 10 μm were successfully produced by solvent evaporation technique, gene expression was not observed with them either.

# CONCLUSION

The microparticles produced by spray drying techniques enabled the production of spherical shape microparticles ranging in size from 0.18 to 20 μm. The optimal conditions to produce microparticles was found to be a solution feed rate of 30 ml/hr, atomized air pressure 15 psi, the air pressure from fluid bed set at 50% of full capacity, and the inlet- outlet drying temperature set at 60-40 °C, respectively. Solvent evaporation technique produced microparticles with a size range from 2-10 μm. The size distribution profiles of microparticles showed that microparticles produced by the spray drying technique produced a log-normal size distribution or skewed size distribution. The study on the degree of swelling showed that the mean diameter of spray-dried microparticles after suspending in simulated gastric fluid (pH 1.23) increased with time and reached a plateau level at about 90 minutes with the approximate diameter of 4.685 μm, which was 80.89 % larger than the diameter of the original dried microparticles, 2.59 μm.

Lowry's assay showed that the ratio of 7:1.5 polymer to drug allowed for the maximum encapsulation efficiency, 100 % into the microparticles. Eudragit® L-30 D, HPMCP-55, and CAP all proved to be good candidates for the preparation of the microparticulate oral vaccine. Eudragit® L-30 D was preferred because of its enteric coated properties along with it being less sticky. As a pH adjusting agent, sodium hydroxide was preferred as a result of smooth surface microparticles obtained. Additionally when ammonium hydroxide was used as a pH adjusting agent, under the

spray drying processes, ammonium evaporated away leaving the DNA in an acidic environment leading to instability of plasmid DNA. To maintain structural and functional integrity of DNA, the optimum storage condition for spray-dried microparticles was found to be 4 °C in dessicator. Light did not have an affect on the stability of pEGFP-N1.

Gene expression could not be observed in Caco-2 cells transfected with either dry spray-dried microparticles or a suspension of microparticles. The need for additional carriers in the microparticles such as liposomes and other cationic reagents to facilitate cell uptake of the DNA appears to be required. The next chapter will discuss the potential use of liposomes and polyethylenimine to induce DNA cells transfection.

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# Chapter 3

## Use of Liposome and Cationic Polymer as Non-Viral Vectors for Gene Delivery into Caco-2 and CHSE-214 Cells

**Angkana Tantituvanont, Benjamin E. Simon, Joann C. Leong, Rosita J. Rodriguez, and J. Mark Christensen**

## ABSTRACT

The development of an oral DNA vaccine in fish is limited by its extensive negative charge, the large molecular weight, and the instability of the DNA in gastrointestinal environment. Liposomes and cationic polymers have been shown to be effective vectors for gene transfer both *in vitro* and *in vivo*. Successful gene delivery with these vectors is believed to be due to their ability to prevent DNA from undergoing enzymatic degradation as well as their binding affinity to the surface of the cell. This study investigates the use of liposomes and polyethylenimine (PEI) as carrier vectors to improve gene transfer into cancer adenocarcinoma cells (Caco-2 cells) and Chinook salmon embryonic cells (CHSE-214 cells). Caco-2 cells were chosen as a model to represent an intestinal epithelial cell and CHSE-214 cells to represent fish cells. Transfection efficiency of these vectors was evaluated by lipid compositions, carrier to DNA charge ratio, and stability of DNA-complexes against DNase. Liposomes were prepared by the thin film technique using natural phospholipids, [1, 2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol (CHO)], polycationic lipids [1, 2-dioleoyl-3-(trimethylammonium)-propane (DOTAP)], and the neutral helper lipid [dioleoylphosphatidylethanolamine (DOPE)]. Different vector formulations were complexed with plasmid DNA encoded with luciferase reporter gene (pCMV-LUC). The compositions of the formulations were: DSPC/CHOL, DSPC/DOTAP/CHOL, DOTAP/DOPE, PEI. Stability studies of DNA complexed with DOTAP/DOPE and PEI was performed with DNase 1. The results show that luciferase expression level increased as the carrier to DNA charge ratio

increased. Optimum conditions cellular uptake of DNA occurred with the positively charged DNA-complexes when compared to less positively or negatively charged DNA-complexes; (PEI > DOTAP/DOPE > DSPC/DOTAP/CHOL). Transfection activity was reduced when DOPE was replaced with DSPC. The DNA complexed with PEI and with DOTAP/DOPE was still intact after 30 minutes exposure to DNase, while non-complexed DNA or naked DNA was degraded within 2 minutes as determined by agarose gel electrophoresis. Luciferase expression level in Caco-2 and CHSE-214 cells transfected with the complexes of DNA and DOTAP/DOPE indicated no statistical significant difference in luciferase activity between cells treated in the presence and the absence of DNase (p-value > 0.869 for Caco-2 cells and p-value > 0.146 for CHSE-214 cells). Cells transfected with complexes of DNA and PEI in the presence of DNase showed a lower luciferase expression level of $15\text{-}10^4$ fold compared to cells transfected in the absence of DNase (p-value > 0.016 for Caco-2 cells and p-value > 0.012 for CHSE-214 cells) suggesting a possible competitive binding of EDTA to PEI. Cells transfected with polyplexes and lipoplexes in the presence of $MnCl_2/MgCl_2$/EDTA showed a higher luciferase expression level of 10-144 fold and 6-10 fold, respectively compared to cells treated in the absence of $MnCl_2/MgCl_2$/EDTA. Transfection efficiency of liposomes and PEI depends on the lipid composition, carrier to DNA charge ratio, and stability of DNA-complexes against DNase. Complexation of DNA with DOTAP/DOPE and with PEI was resistant to DNase degradation, although some portions of DNA in the complexes were digested as determined by cell transfection assay and agarose gel electrophoresis.

PEI and liposomes composed of DOTAP/DOPE are efficient vectors in deliver DNA into both Caco-2 and CHSE-214 cells. Transfection efficiency of PEI and DOTAP/DOPE in deliver DNA into cells can be improved with the use of $MnCl_2/MgCl_2$/EDTA, however more research is needed to understand the mechanism of $MnCl_2/MgCl_2$ and EDTA on cellular uptake of DNA. Thus, this study could be of significant importance in optimizing and developing oral delivery systems for macromolecule.

# INTRODUCTION

Oral immunization of plasmid DNA is limited by the extensive negative surface charge, the large size (large molecular weight) (Guo and Lee, 2000), and the stability of the DNA in gastrointestinal tract environment. Previous studies from our laboratory reported that DNA or protein embedded into microparticles produced using an enteric-coated polymer, Eudragit® L-30 D, protected the DNA from the acidic environment of the gastrointestinal tract (Zhang, 1995). In addition, intracellular uptake of DNA is not only limited by the size of microparticles obtained but also the characteristics of DNA itself which possesses a large size (6 million dalton) and an abundance of negative charge.

Several attempts have been made to develop efficient vectors for gene delivery into eukaryotic cells. Guo and Lee, 2000 reported the use of a synthetic vector based on polyplexes (poly-L-lysine) complexed to anionic liposomes (dioleoylphosphatidylethanolamine and cholesteryl hemisuccinate) in delivery of DNA into human oral carcinoma KB cells. Sen et al., 2001 investigated the use of adenovirus and cationic liposomes in mediated gene therapy in cardiac allografts. Fennelly et al., 1999 reported the use of bacterial vectors (*Shigella flexneri*) to deliver a measles virus DNA vaccine in a mice (Fennelly et al., 1999).

In general, gene delivery can be classified into two systems, viral and non-viral systems. High transfection efficiency has been observed with viral vectors such as adenovirus (Sen et al., 2001). The risk of immunogenicity and pathogenicity, however, has restricted their use (Pleyer et al., 2001). Non-viral vectors are less

immunogenic and not as efficient as viral vectors (Guo and Lee, 2000). Among non-viral vectors, particular attention has been given to the use of liposomes and cationic polymers because of their ease in preparation, high carrier capacity, and ease for scale up for production (Han et al., 2001).

Cationic compounds such as polyethylenimine (PEI) and liposomes composed of 1,2-dioleoyl-3-(trimethylammonium)-propane (DOTAP) have been shown to be effective carriers for gene delivery into several different cell cultures such as ovarian adenocarcinoma cells (Kim et al., 2002), murine, and human hepatocyte-derived cells (Zanta et al., 1997). Successful gene delivery with these vectors is believed to be due to their ability to tightly condense plasmid DNA into small particles as well as their binding affinity to the surface of the cell.

Cationic polymers and cationic liposomes form complexes with the negative charged portion of the DNA molecule resulting in "polyplexes" and "lipoplexes", respectively. These positively charged complexes are internalized into endosomes via an endocytosis pathway. Theoretically, once endocytosis occurs, contents in endosomes will be transferred to the lysosomes and degraded by lysosomal enzymes (Ropert, 1999). For effective gene delivery, the plasmid DNA needs to survive the degradation in the lysosomes, be released into the cytosol, and subsequently be expressed in the nucleus (Figure 3.1 and 3.2). Dioleoylphosphatidylethanolamine (DOPE) and PEI have been proposed as membrane destabilizing agents which can facilitate endosomal escape (Godbey et al., 2000; Oku et al., 2001).

DOPE is often used as a helper lipid in cationic liposomal formulations to provide membrane fusogenicity (Guo and Lee, 2000). DOPE facilitates endosomal escape by destabilizing endosomal membranes resulting in a release of genetic materials in the cytosol before they reach the lysosomes (Farhood et al., 1995).

**Figure 3.1: Hypothetical schemes of gene transfer into eukaryotic cells by cationic liposomes composed of DOPE.** (1) Liposome/DNA complexes enter the eukaryotic cell by endocytosis; (2) The complexes is internalized into endosome; (3) Fusion of liposomes to endosomal membrane leading to membrane disruption; (4) Plasmid DNA is released into the cytoplasm of the eukaryotic cell; (5) Entering of plasmid DNA in nucleus.

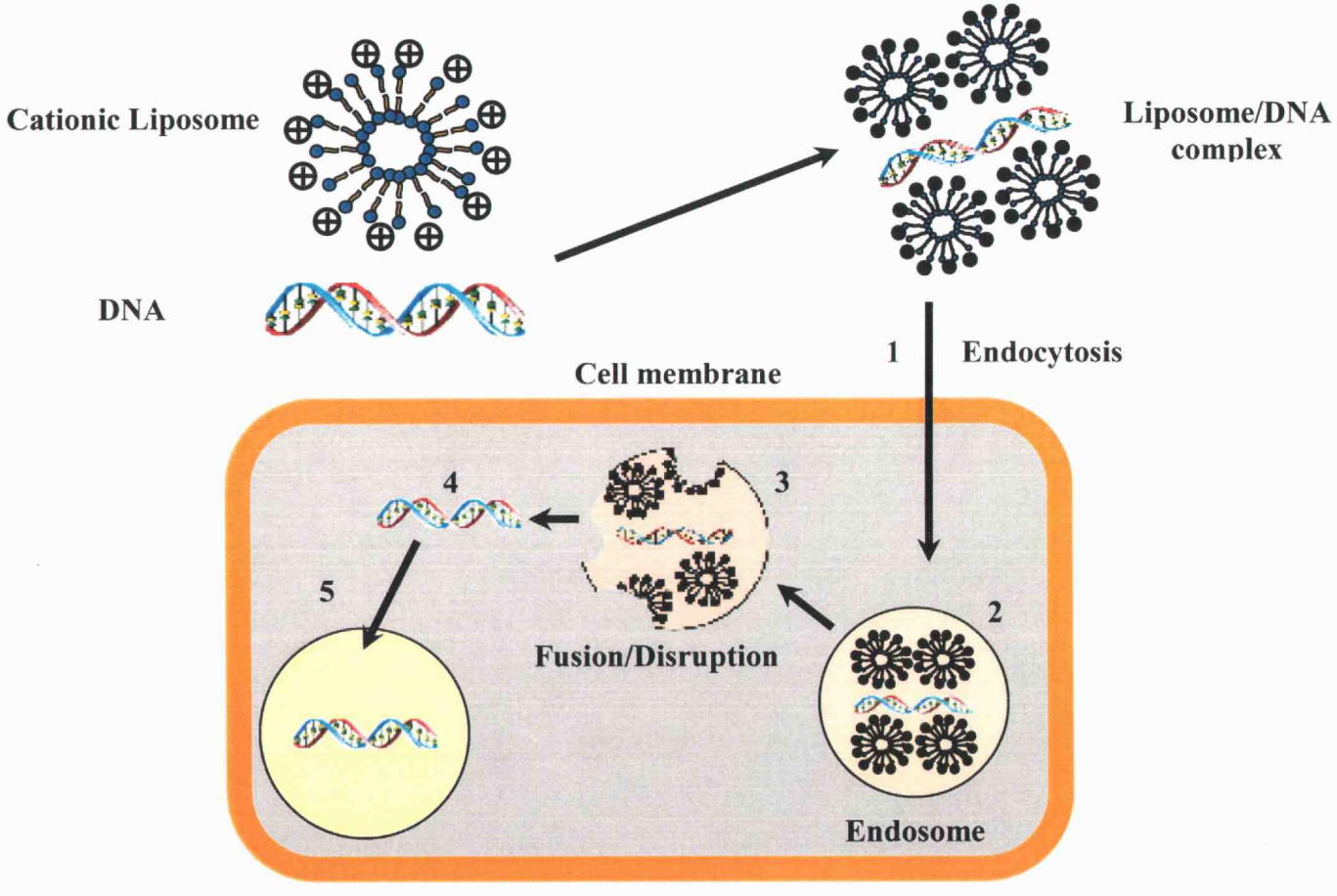

Cationic Liposome
DNA
Liposome/DNA complex
1
Endocytosis
Cell membrane
2
3
4
5
Fusion/Disruption
Endosome

**Figure 3.1:**

PEI is an organic macromolecule possessing a high cationic charge density. Every third atom is an amine that can be partially protonated at physiological pH (Boussif et al., 1995). Upon acidification within the endosomes, PEI acts as a proton sponge that induces osmotic swelling and rupture of endosomal membrane which provides an escape mechanism for plasmid DNA-complexes (Godbey et al., 2000).

The oral administration of DNA is usually limited by their inefficient transport across the gastrointestinal membrane. Thus, it is very important to investigate the factors that affect gastrointestinal uptake of DNA-complexes in order to optimize and develop effective oral delivery systems for macromolecules. In this study, human adenocarcinoma cells (Caco-2 cells) and Chinook salmon embryonic (CHSE-214) cells were chosen as models to represent intestinal epithelial cells and fish cells, respectively. Caco-2 cells are widely used as an *in vitro* model for absorption studies (Artursson, 1989; Desai et al., 1997; Tomita et al., 1996). Caco-2 cells demonstrate morphological and several functional characteristics of human small intestinal epithelium.

"They form tight junctions, develop microvilli, express brush border enzymes, and express growth factor receptors" (Delie and Rubas, 1997).

These characteristics make Caco-2 cells a good *in vitro* model for the study of gastrointestinal uptake of DNA. Additionally, this study is to investigate effective vectors for the development of oral delivery system for fish. Thus, CHSE-214 cells, an epithelial cell line derived from Chinook salmon (*Oncorhynchus tshawytscha*)

embryos were also used as a tool to investigate the uptake efficiency of DNA-complexes in fish cells.

This study investigates the use of liposomes and polyethylenimine (PEI) as carrier vectors to improve gene transfer into the Caco-2 and CHSE-214 cells. Transfection efficiency of these vectors was evaluated by lipid compositions, carrier to DNA charge ratio, and stability of DNA-complexes against DNase.

**Figure 3.2: Hypothetical schemes of gene transfer by polyethylenimine (PEI).** (1) PEI/DNA complexes enter the eukaryotic cell by endocytosis; (2) The complexes is internalized into endosome; (3) Osmotic swelling of PEI causing a rupture of endosomal membrane; (4) Plasmid DNA is released into the cytoplasm of the eukaryotic cell; (5) Entering of plasmid DNA in nucleus.

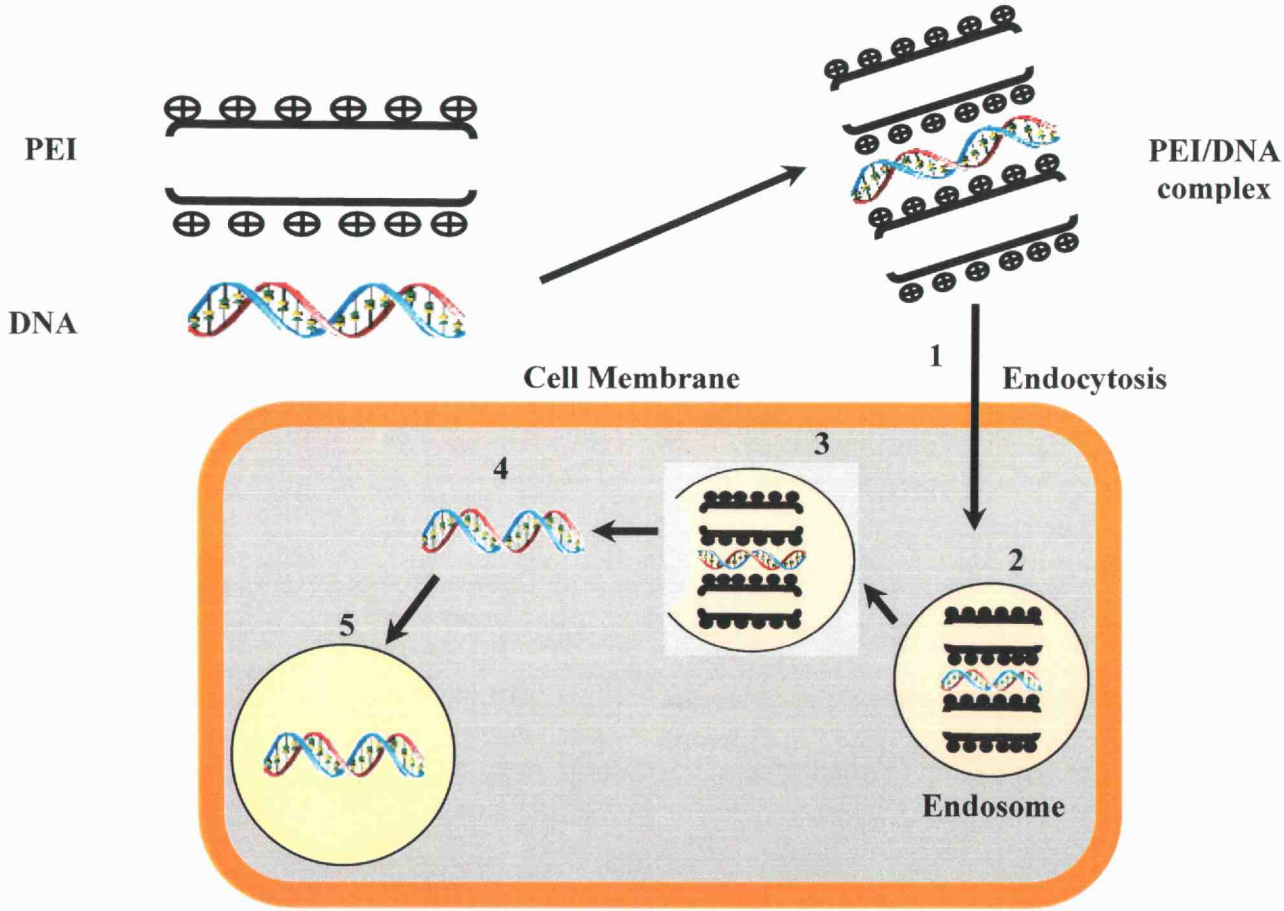

PEI
DNA
PEI/DNA
complex
1
Endocytosis
Cell Membrane
2
3
4
5
Endosome

Figure 3.2

## MATERIALS AND METHODS

### Polymers and Additives

1, 2-distearoyl-sn-glycero-3-phosphocholine (DSPC, MW 790.15), dioleoylphosphatidylethanolamine (DOPE, MW 744.04), 1, 2-dioleoyl-3-(trimethylammonium)-propane (DOTAP, MW 698.55) were purchased from Avanti Polar-Lipids, Inc. (Alabaster, AL, USA). Lecithin was obtained from Spectrum, Spectrum Quality Products, Inc. (Gardena, CA, USA). Cholesterol (CHO, MW 386.7), polyethylenimine (PEI, 50% w/v) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Chloroform was obtained from Fisher Chemical (Fair Lawn, NJ, USA).

### Model Drugs

Bovine serum albumin (BSA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Plasmid DNA encoded with a fluorescence reporter gene (pEGFP-N1) and a firefly luciferase reporter gene (pCMV-LUC) under the control of cytomegalovirus promoter were gifts from Dr. Joann Leong, Department of Microbiology, Oregon State University (Corvallis, OR, USA).

"The reporter plasmid pLUC was constructed by inserting the 2.7 kb BamHI/HindIII fragment (containing the luciferase gene) of pGL2 basic, Promega (Madison, WI, USA) into BamHI/HindIII digested pCDNA3, Invitrogen (Carlsbad, CA, USA) (Simon, 2001.)"

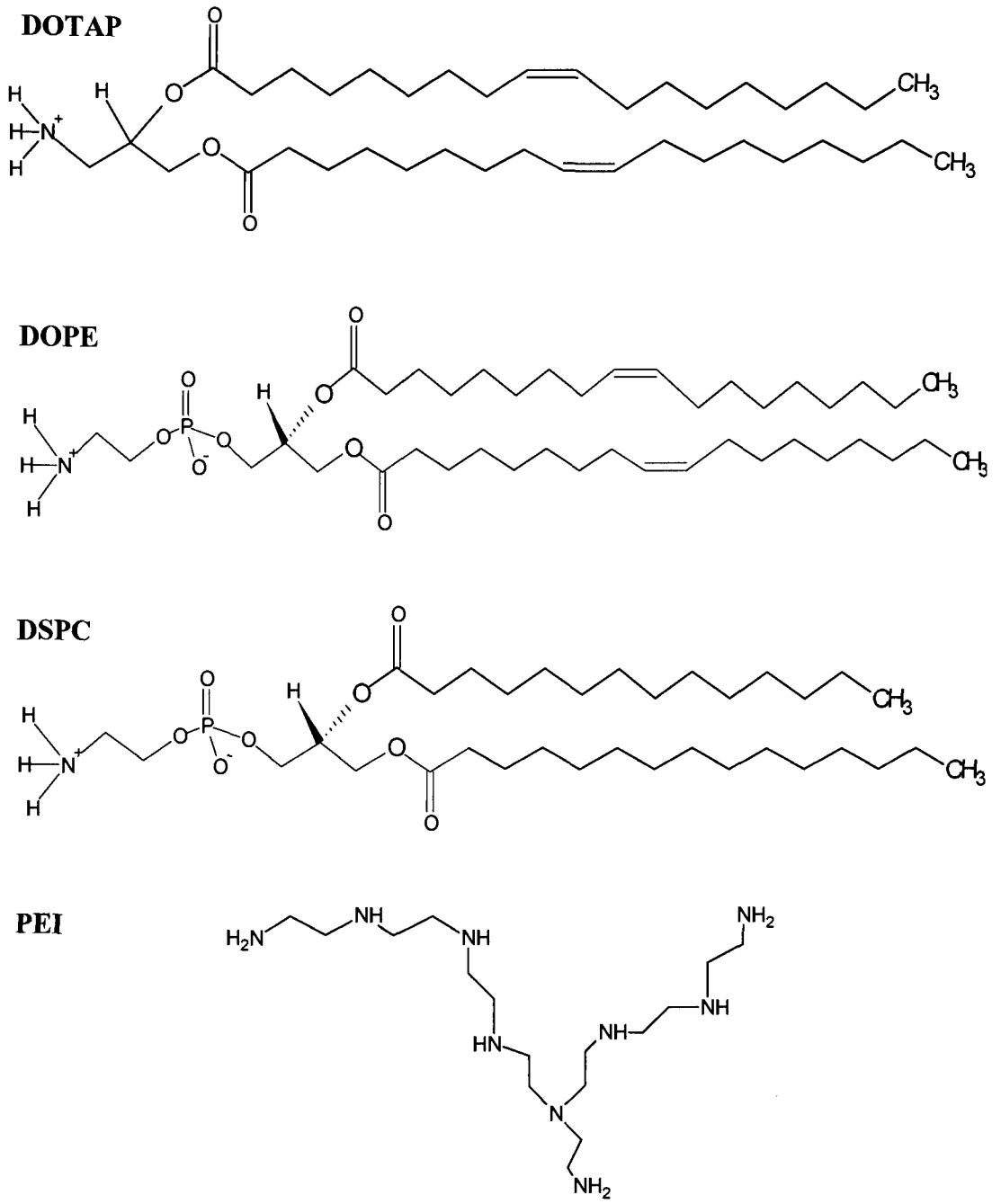



**Figure 3.3: Chemical structure of lipid ingredients;** DOTAP, DOPE, DSPC, and PEI.

**Cell Culture**

Two types of cell lines were used in this experiment, human adenocarcinoma cell line (Caco-2 cells) was a gift from Dr. Rosita Rodriguez, Oregon State University (Corvallis, OR, USA) and Chinook salmon embryonic cells line (CHSE-214 cells) as a gift from Dr. Joann Leong, Department of Microbiology, Oregon State University (Corvallis, OR, USA).

Human adenocarcinoma cell line (Caco-2 cells) were maintained in Dulbecco's modified eagle medium (DMEM), Life Technologies Inc. (Rockville, MD, USA) supplemented with 10% fetal bovine serum, SUMMIT Biotechnology (Fort Collins, CO, USA), 1% MEM non-essential amino acid solution, Life Technologies Inc. (Rockville, MD, USA), 1% of 100 nM sodium pyruvate, Sigma Chemical Co. (St. Louis, MO, USA), and 10,000 unit penicillin, 10 mg streptomycin, and 25 μg amphotericin B per ml in 0.9% sodium chloride, Sigma Chemical Co. (St. Louis, MO, USA ) in a 1:100 ratio of antibiotic antimycotic solution to medium. The pH of medium was maintained at 7.4 by buffering with sterile sodium bicarbonate solution, Sigma Chemical Co. (St. Louis, MO, USA). Caco-2 cells were grown and maintained in T 75 $cm^2$ tissue culture flasks at 37 °C in 5% $CO_2$ humidified atmosphere. Cells were used for experiments between passages 25 and 40.

Chinook salmon (*Oncorhynchus tshawytscha*) embryonic cell line (CHSE-214 cells) was maintained in autoclaved minimum essential media (MEM), Life Technologies Inc. (Rockville, MD, USA) supplemented with 5 % fetal bovine serum

(Intergen # 1020-01) and 1% of 200 nM L-Glutamine, Life Technologies Inc. (Rockville, MD, USA). The pH of medium was maintained at 7.4 by buffering with sterile 7.5% sodium bicarbonate solution, Sigma Chemical Co. (St. Louis, MO, USA). CHSE-214 cells were grown and maintained in T 75 $cm^2$ tissue culture flasks at 17 °C.

## I. Preparation of Liposomes

Reverse Phase Evaporation Method

Liposomes containing model drugs, BSA or pEGFP-N1, were prepared according to formulations and method developed by Sorasuchart, (1998), Table 3.1. Briefly, lipid ingredients (DSPC, lecithin, and CHO) were dissolved in 5 ml of chloroform. Five ml of sterile phosphate buffer saline (PBS) containing model drug in a concentration of 1 mg/ml was added into the organic solution followed by vortexing until an emulsion formed. The resultant emulsion was then sonicated for 10 minutes and then vortexed for 1 minute. This process was repeated three times followed by evaporation of chloroform under vacuum using rotavapor, Flash Evaporator, Buchler Instruments (Fort Lee, NJ, USA) at 37 °C. The liposomes suspension was vortexed and extruded 21 times through 800 nm polycarbonate filters at room temperature using an extrusion device, Liposofast®, Avetin, Inc. (Ottawa, ON, Canada).

**Table 3.1: Liposome formulations for reverse phase evaporation method**

| Formulation | Ingredients | Amount (g) | Conc. (μM) | Mole ratio |
|---|---|---|---|---|
| 1 | Lecithin (g) | 0.1740 | - | - |
| | CHO (g) | 0.0260 | 67 | - |
| | BSA (1 mg/ml) | 5 | | |
| | $CHCl_3$ (ml) | 5 | | |
| 2 | Lecithin (g) | 0.0830 | - | - |
| | CHO (g) | 0.0130 | 34 | - |
| | BSA (1 mg/ml) | 5 | | |
| | $CHCl_3$ (ml) | 5 | | |
| 3 | DSPC (g) | 0.1740 | 220 | 3.3 |
| | CHO (g) | 0.0260 | 67 | 1 |
| | BSA (1 mg/ml) | 5 | | |
| | $CHCl_3$ (ml) | 5 | | |
| 4 | DSPC (g) | 0.0830 | 105 | 3.1 |
| | CHO (g) | 0.0130 | 34 | 1 |
| | BSA (1 mg/ml) | 5 | | |
| | $CHCl_3$ (ml) | 5 | | |
| 5 | DSPC (g) | 0.1740 | 220 | 3.3 |
| | CHO (g) | 0.0260 | 67 | 1 |
| | pEGFP-N1 (1 mg/ml) | 5 | | |
| | $CHCl_3$ (ml) | 5 | | |
| 6 | DSPC (g) | 0.0830 | 105 | 3.1 |
| | CHO (g) | 0.0130 | 34 | 1 |
| | pEGFP-N1 (1 mg/ml) | 5 | | |
| | $CHCl_3$ (ml) | 5 | | |

## Determination of BSA and pEGFP-N1 Content in Liposomes Prepared by Reverse Phase Evaporation Method

### BSA Content of Liposomes

To determine the amount of BSA entrapped in liposomes, half ml of liposomes suspension was centrifuge at 14,000 rpm (10955 ×g) using Eppendorf Centrifuge 5415C, Brinkmann Instruments, Inc. (Westbury, NY, USA) for 5 minutes and the supernatant was discarded. Liposomes were washed three times with 0.5 ml sterile PBS followed by vortexing and centrifugation to remove unentrapped BSA. Liposome pellets were ruptured by adding 100 µl of isopropyl alcohol and followed by vigorous vortexing. The amount of encapsulated BSA was measured using Lowry's protein assay.

### Reagent for Lowry's Protein Assay

Reagent $A_1$, 2% sodium tartrate, was prepared by dissolving 2.0 g of sodium tartrate, $Na_2C_4H_4O_6$, Sigma Chemical Co. (St. Louis, MO, USA) in 100 ml of deionized water. Reagent $A_2$, 1% copper sulfate, was prepared by dissolving 1.0 g of copper sulfate, $CuSO_4.H_2O$, Sigma Chemical Co. (St. Louis, MO, USA) in 100 ml of deionized water. Reagent $A_3$ (2% sodium carbonate) was prepared by dissolving 20.0 g of $Na_2CO_3$, Sigma Chemical Co. (St. Louis, MO, USA) in 1 liter of 0.1 N NaOH. Solution A was prepared by mixing $A_1$, $A_2$, and A3 in 0.75:0.75:15 ratio. Reagent was

prepared by adding 5 ml of 2 N Folin & Ciocalteu's phenol reagent, Sigma Chemical Co. (St. Louis, MO, USA) into 50 ml of deionized water (DI water).

**Lowry's Protein Assay**

To 1 ml of dissolution sample containing BSA, 1 ml of reagent A was added. The mixture was mixed well and allowed to stand for 15 minutes at room temperature. After 15 minutes, 3 ml of reagent B was added and mixed very rapidly. The mixture was allowed to stand for another 45 minutes at room temperature followed by measuring the absorbance at the wavelength of 540 nm using a spectrophotometer, Beckman DU-600, Beckman Coulter, Inc. (Fullerton, CA, USA).

**pEGFP-N1 Content of Liposomes**

The procedure for extracting the encapsulated plasmid DNA (pEGFP-N1) from Liposomes was the same as was performed for bovine serum albumin, previously described. To determine the amount of pEGFP-N1 loaded in liposomes, the extracted pEGFP-N1 was further purified by QIAquick PCR purification kit, QIAGEN Inc. (Valencia, CA, USA) followed by measuring the UV absorption at 260 nm using Beckman UV spectrophotometer, Beckman DU-600, Beckman Coulter, Inc. (Fullerton, CA, USA).

Thin film method

Liposome formulations (see Table 3.2) and preparation processes used were modified based on the study of Gregoriadis et al., (1999). Lipid ingredients were completely dissolved in 5 ml of chloroform in a 50 ml round bottom flask. Chloroform was removed under vacuum at 37 °C using rotavapor, Flash Evaporator, Buchler Instruments (Fort Lee, NJ, USA). The thin film obtained was hydrated with 2 ml of sterile DI water followed by hand shaking at 70 °C until a milky suspension was formed. The suspension was left to stand in water bath at 70 °C for 2 hours. Then the milky suspension was sonicated at 70 °C for four cycles, 40 second of sonication followed by a 40 second resting interval, until a clear or slightly opaque solution was formed. Liposomes solution was left to stand in the water bath at 70 °C for 2 hours. Upon completion of the formation of the liposomes they were stored at 4 °C.

**Table 3.2: Liposome formulation for thin film method**

| Formulation | Ingredients | Amount (g) | Conc. (μM) | Mole ratio |
|---|---|---|---|---|
| 7 | DSPC (g) | 0.02528 | 32 | 1 |
| | CHOL (g) | 0.01237 | 32 | 1 |
| | $CHCl_3$ (ml) | 5 | | |
| 9 | DSPC (g) | 0.03792 | 48 | 1.5 |
| | DOTAP (g) | 0.00559 | 8 | 0.25 |
| | CHOL (g) | 0.01237 | 32 | 1 |
| | $CHCl_3$ (ml) | 5 | | |
| 10 | DSPC (g) | 0.02528 | 32 | 1 |
| | DOTAP (g) | 0.00559 | 8 | 0.25 |
| | CHOL (g) | 0.01237 | 32 | 1 |
| | $CHCl_3$ (ml) | 5 | | |
| 11 | DOTAP (g) | 0.02236 | 32 | 1 |
| | DOPE (g) | 0.02381 | 32 | 1 |
| | $CHCl_3$ (ml) | 5 | | |

## II. Cell Transfection

Caco-2 and CHSE-214 cells were seeded in 12 well culture plates at a cell density of $1 \times 10^6$ cells per well 24 hours before transfection. Cells were allowed to attach to the plate surface as a monolayer by growing in DMEM supplemented with 10% FBS at 37 °C under 5% $CO_2$ humidified atmosphere for Caco-2 cells, and by growing in MEM supplemented with 5% FBS, at 17 °C for CHSE-214 cells. Prior to

transfection, cells with 60-70% confluence were washed twice with 1 ml serum free medium (DMEM supplemented with 1% MEM non-essential amino acid, 1% sodium pyruvate) and then covered with 0.8 ml of serum free medium before adding transfection complexes (lipoplexes or polyplexes). Approximately 0.2 ml of transfection complexes were added gently to the cells in each well. Cells were incubated for 3 hours at 37 °C under 5% $CO_2$ humidified atmosphere for Caco-2 cells, and at 17 °C for CHSE-214 cells. After incubation for 3 hours, the transfection medium was removed and replaced with 1 ml of complete medium (DMEM supplemented with 10% FBS, 1% MEM non-essential amino acid, 1% sodium pyruvate, and 10,000 unit penicillin, 10 mg streptomycin, and 25 μg amphotericin B per ml in 0.9% sodium chloride in a 1:100 ratio of antibiotic antimycotic solution to medium), and cells were incubated for at least 72 hours. Transfection was performed in triplicate with liposome formulations 9, 10, and 11 and PEI, Table 3.2. Cells treated with DNA/lipofectin® complexes served as a positive control.

**Preparation of Liposome/DNA Complexes (Lipoplexes)**

Lipoplexes were prepared according to the Promega's protocol with Lipofectin®. Briefly, plasmid DNA and liposomes were each diluted in 100 μl of serum free medium and allowed to incubate at room temperature for 15 minutes. Plasmid DNA solution was then added at once into the liposome solution and gently

mixed. The mixture was further incubated at room temperature for 30-45 minutes before adding to the cells.

**Preparation of PEI/DNA Complexes (Polyplexes)**

The procedure for preparing polyplexes was the same as with lipoplexes except that 150 nM of sterile sodium chloride solution was used instead of serum free medium in the dilution process.

**Luciferase Assay**

Luciferase gene expression was examined by measuring the light production of luciferase using luciferase assay system protocol, Promega (Madison, WI, USA). Briefly, the transfected cells were washed twice with phosphate buffer saline (PBS). Cells in each well were lysed with 100 µl of 1X lysis buffer, Promega (Madison, WI, USA), scraped, and the lysates were transferred to microcentrifuge tubes. Cells lysates were assayed for luciferase activity with luciferase assay reagent, Promega (Madison, WI, USA) in a Lumat LB9507 luminometer, EG&G Berthold. (Wilbach, Germany). To 20 µl of cell lysates, 100 µl of luciferase assay reagent is added. The protein content of the lysates was measured using Bio-Rad DC protein assay, Bio-Rad Laboratories. (Hercules, CA, USA). Luciferase activity was expressed as relative light units (RLUs) per mg of total cell protein.

**Effect of Carrier to DNA Charge ratio on Gene Transfer to Cells**

Plasmid DNA encoded with luciferase gene under the control of cytomegalovirus promoter (pCMV-LUC) was used as a reporter gene. The effect of carrier to DNA charge ratio on gene transfer into cells was evaluated by the change in luciferase activity obtained with cells transfected with liposome/DNA complexes (lipoplexes) and with PEI/DNA complexes (polyplexes) at varying carrier to DNA charge ratios. Carrier to DNA charge ratio was determined as cationic lipid/DNA charge ratio (charge of the nitrogen atoms in DOTAP versus charge of phosphate in DNA) for liposomes and N/P ratio (charge of the nitrogen atoms in PEI versus charge of phosphate in DNA) for PEI. It is note that 1 μg DNA is 3 nM of phosphate, 1 μl of PEI stock solution (0.9 mg/ml) contain 10 nM of amine nitrogen, and 1 μl of liposome 9, 10, and 11 stock solution (1 mg/ml) contain 0.143205, 0.185067, 0.693289 nM of amine nitrogen, respectively. Liposomes were produced according to formulations 9, 10, and 11 using thin film method, Table 3.2. Both Caco-2 and CHSE-214 cells were employed in cell transfection experiments. The amount of DNA was 1.5 μg in all tested experiments while the amount of liposomes and PEI were varied according to Table 3.3. Carrier to DNA charge ratio that yielded the highest luciferase expression level was chosen for further study.

**Table 3.3: The amount of liposomes and PEI along with their DNA[a] charge ratio for cells transfection**

| Ingredients | Amount (μl) | Amount (μg) | Lipid/DNA or N/P charge ratio[b] |
|---|---|---|---|
| Liposomes 9 (1mg/ml) | 25 | 25 | 0.8 |
| | 30 | 30 | 0.96 |
| | 35 | 35 | 1.12 |
| | 40 | 40 | 1.28 |
| | 45 | 45 | 1.44 |
| Liposomes 10 (1mg/ml) | 15 | 15 | 0.617 |
| | 20 | 20 | 0.822 |
| | 25 | 25 | 1.028 |
| | 30 | 30 | 1.233 |
| | 35 | 35 | 1.439 |
| Liposomes 11(1mg/ml) | 2 | 2 | 0.308 |
| | 4 | 4 | 0.615 |
| | 8 | 8 | 1.23 |
| | 12 | 12 | 1.845 |
| | 18 | 18 | 2.768 |
| | 24 | 24 | 3.691 |
| PEI (0.9 mg/ml) | 2 | 1.8 | 4.44 |
| | 4 | 3.6 | 8.89 |
| | 8 | 7.2 | 17.78 |
| | 12 | 10.8 | 26.67 |
| | 18 | 16.2 | 40 |

[a]The amount of DNA was 1.5 μg in all experiments.
[b]N/P ratio is charge of the nitrogen atoms in PEI or DOTAP versus charge of phosphate in DNA.

**Effect of Vector Type and Lipid Compositions on Gene Transfer to Cells**

To determine the effect of vector type and lipid compositions on gene transfer into cells, polycationic polymer (PEI) and cationic liposomes produced according to formulations 9, 10, and 11 were used in cell transfection. The amount of DNA used was 1.5 μg, the amount of carrier vector (liposomes or PEI) used was defined as optimal conditions as shown in Table 3.4.

**Table 3.4: Optimal amount of liposomes and PEI for cells transfection**

| Optimal Conditions | | | |
|---|---|---|---|
| **Ingredients** | **Amount (μl)** | **Amount (μg)** | **Lipid/DNA[a] or N/P charge ratio[b]** |
| Liposomes 9 (1mg/ml) | 30 | 30 | 0.96 |
| Liposomes 10 (1mg/ml) | 25 | 25 | 1.028 |
| Liposomes 11(1mg/ml) | 12 | 12 | 1.845 |
| PEI (0.9 mg/ml) | 4 | 3.6 | 8.89 |

[a]The amount of DNA was 1.5 μg in all experiments.
[b]N/P ratio is charge of the nitrogen atoms in PEI or DOTAP versus charge of phosphate in DNA.

**Stability of the DNA Complexes against DNase 1**

The protective effect of cationic polymers (PEI) and cationic liposomes (DOTAP/DOPE, liposome formulation 11) on DNA degradation by DNase was evaluated with agarose gel electrophoresis and cell transfection using the method developed by Moret et al., (2001). Briefly, 460 μl of solution containing 25 μg/ml of naked DNA or DNA complexes was added to 52 μl of solution containing 35 mM of magnesium chloride ($MgCl_2$) and 35 mM of manganese chloride ($MnCl_2$). At time zero, 56 μl of solution was withdrawn and 65 μl of Dnase 1 solution (1mg/ml, 2200 units/mg) was added to the mixture. The mixture was incubated at 37 °C and samples of 64 μl were taken at 2, 5, 10, 15, 20, 25, and 30 minutes. Three microliters of 0.5 M EDTA was immediately added to each collected sample to stop DNase degradation and samples were then placed in an ice bath. All samples were subjected to agarose gel (0.6%) electrophoresis, after adding heparin.

In the cell transfection studies, 1.5 μg of DNA was complexed with either 4 μl of PEI (0.9 mg/ml) or 12 μl of DOTAP/DOPE (1 mg/ml). The DNA complexes were added to 5 μl of solution containing 35 mM of magnesium chloride and 35 mM of manganese chloride followed by 6 μl of Dnase 1 solution (1mg/ml, 2200 units/mg). The mixture was incubated at 37 °C for 15 minutes. Three microliters of 0.5 M EDTA was immediately added to the sample to stop DNase degradation and samples were then placed in an ice bath for 5 minutes. All samples were used immediately after their preparation for the cell transfection studies.

**Agarose Gel Electrophoresis**

The samples were mixed with 5 µl gel loading buffer (bromophenol blue 0.05% w/v, sodium dodecyl sulphate 0.05% w/v, sucrose 40% w/v, EDTA 0.1 M pH 8) and subjected to agarose gel electrophoresis in the presence of ethidium bromide for 10 minutes at 210 Volt. DNA visualization of the gels was carried out under UV lamp, Fotodyne (New Berlin, WA, USA).

## RESULTS AND DISCUSSION

### Preparation of Liposomes

A large agglomeration of lipids was observed with liposome formulation 1 and 2 composed of lecithin and CHOL (Table 3.1) which were prepared by reverse phase evaporation method. Replacing lecithin with DSPC in liposome formulation 3 and 4 resulted in better a production method for liposomes. No large agglomeration of lipids was observed but the amount of encapsulated drug was undetectable. Liposomes of larger particle size were always visible. The size comparison of the liposomes can be approximately determined by visualization of the opaqueness of liposome solution, the clearer the solutions of liposomes the smaller the particle size. Thin film method allowed the preparation of smaller size liposomes especially when DOTAP and DOPE were included in the liposome formulation. The size of liposomes was ranked as DSPC/CHOL > (48) DSPC/DOTAP/CHOL > (32) DSPC/DOTAP/CHOL > DOTAP/DOPE, (liposome formulation 7 > liposome formulation 9 > liposome formulation 10 > liposome formulation 11), Table 3.2. Note that (48) DSPC/DOTAP/CHOL and (32) DSPC/DOTAP/CHOL represents liposome formulations composed of 48 μM and 32 μM of DSPC, respectively.

## Cell Transfection Experiment

### Effect of Carrier to DNA Charge ratio on Gene Transfer to Cells

Gene expression was observed in both Caco-2 and CHSE-214 cells transfected with either DNA/PEI complexes (polyplexes) or with the complexes of DNA and liposomes (lipoplexes) composed of DOTAP (liposome formulations 9, 10, and 11). Luciferase expression level was found to increase with an increase in the charge ratio of carrier to DNA as determined by cationic lipid/DNA charge ratio for liposomes and N/P ratio for PEI (Figure 3.4-3.7). Because gene transfer into cells depends on the electrostatic interaction between DNA-complexes and the cells surface (Zou et al., 2000), increasing the electrostatic interactions of the positive charged DNA-complexes with the negatively charged cell membrane by increasing carrier to DNA charge ratio will consequencely enhance gene transfer (Boussif et al., 1999; Guo and Lee, 2000). The optimum cationic lipid/DNA charge ratio was found to be 0.96, 1.028, and 1.845 for (48) DSPC/DOTAP/CHOL, (32) DSPC/DOTAP/CHOL, and DOTAP/DOPE, respectively, while the optimum N/P ratio of PEI was founded to be 8.89, Table 3.9. A further increase in the carrier to DNA charge ratio results in the reduction of luciferase expression possibly due to cytotoxicity from the high concentration of lipids or polymers as visualize changes in the cells morphology occurred (cell shrinkage and detachment from the culture plate). Guo and Lee, 2000

reported that complexes of PEI and DNA at a N/P ratio below 10 was relatively nontoxic while cytotoxicity was observed at N/P ratios higher than 15.

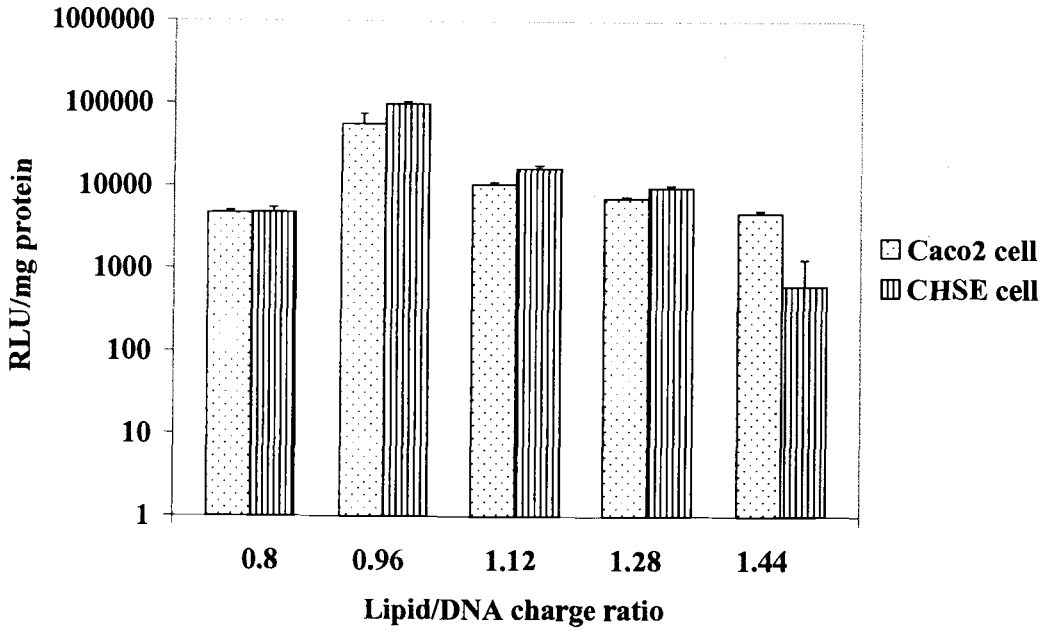



**Figure 3.4: Cell transfected with complexes of liposome formulation 9 and plasmid pCMV-LUC.** Caco-2 cells and CHSE-214 cells were transfected with pCMV-LUC (1.5 μg per well) at 0.8-1.44 lipid/DNA charge ratio. Relative light units (RLU) were normalized to 1 mg of cell protein. Transfection efficiency is maximal at 0.96 lipid/DNA charge ratio. Mean ± SD (n=3).

**Table 3.5: Cell transfected with complexes of liposome formulation 9**

| Lipid/DNA charge ratio | RLU/mg protein | | | |
|---|---|---|---|---|
| | Caco-2 cells | SD | CHSE-214 cells | SD |
| 0.8 | 4,730 | 334 | 4,802 | 668 |
| 0.96 | 56,902 | 19,565 | 98,676 | 6,521 |
| 1.12 | 10,648 | 752 | 16,620 | 1,505 |
| 1.28 | 7,255 | 344 | 9,657 | 688 |
| 1.44 | 4,801 | 339 | 620 | 678 |

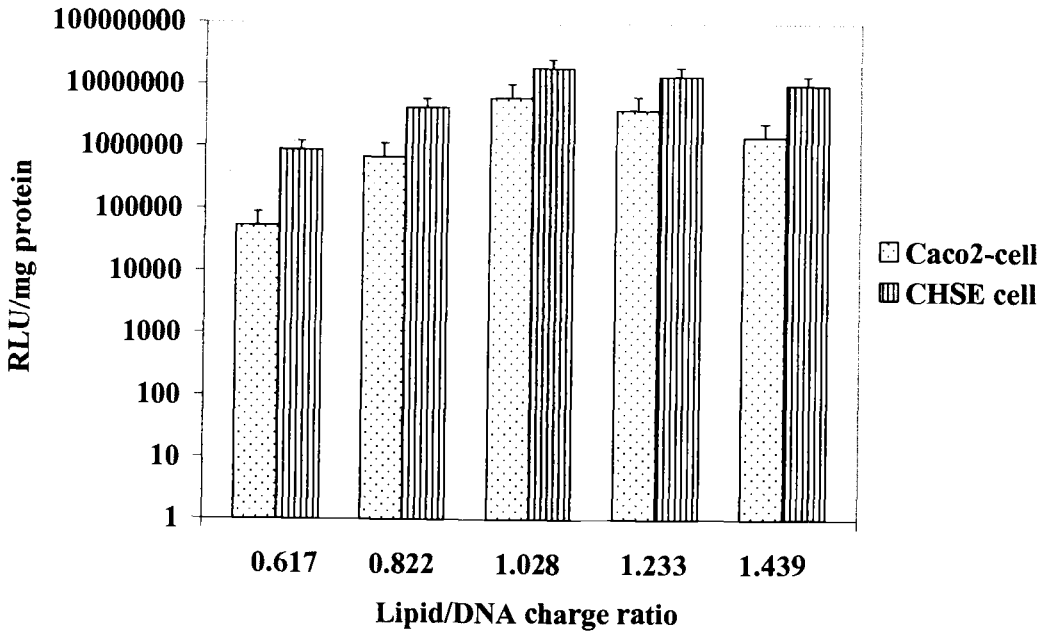



**Figure 3.5: Cell transfected with complexes of liposome formulation 10 and plasmid pCMV-LUC.** Caco-2 cells and CHSE-214 cells were transfected with pCMV-LUC (1.5 µg per well) at 0.617-1.439 lipid/DNA charge ratio. Relative light units (RLU) were normalized to 1 mg of cell protein. Transfection efficiency is maximal at 1.028 lipid/DNA charge ratio. Mean ± SD (n=3).

**Table 3.6: Cell transfected with complexes of liposome formulation 10**

| Lipid/DNA charge ratio | RLU/mg protein | | | |
|---|---|---|---|---|
| | Caco-2 cells | SD | CHSE-214 cells | SD |
| 0.617 | 52,532 | 35,022 | 887,147 | 354,858 |
| 0.822 | 692,619 | 461,746 | 4,303,853 | 1,721,541 |
| 1.028 | 6,279,241 | 4,186,160 | 19,287,752 | 7,715,100 |
| 1.233 | 3,961,034 | 2,640,690 | 13,967,279 | 5,586,911 |
| 1.439 | 1,456,289 | 970,860 | 10,037,383 | 4,014,953 |

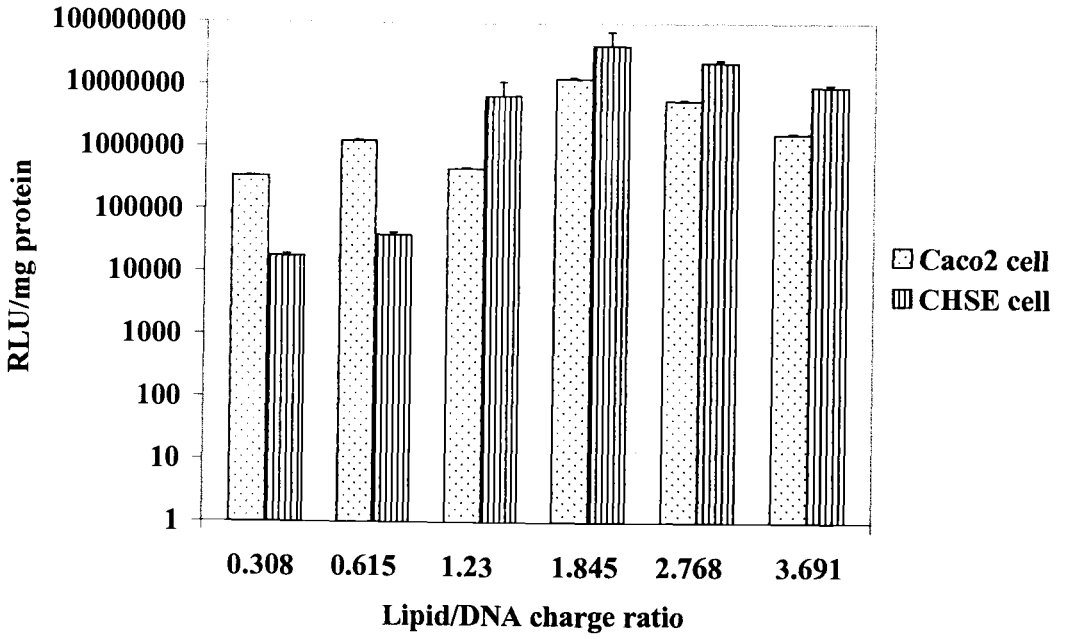



**Figure 3.6: Cell transfected with complexes of liposome formulation 11 and plasmid pCMV-LUC.** Caco-2 cells and CHSE-214 cells were transfected with pCMV-LUC (1.5 µg per well) and 0.308-3.691 lipid/DNA charge ratio. Relative light units (RLU) were normalized to 1 mg of cell protein. Transfection efficiency is maximal at 1.845 lipid/DNA charge ratio. Mean ± SD (n=3).

**Table 3.7: Cell transfected with complexes of liposome formulation 11**

| Lipid/DNA charge ratio | RLU/mg protein | | | |
|---|---|---|---|---|
| | Caco-2 cells | SD | CHSE-214 cells | SD |
| 0.308 | 335,654 | 11,188 | 17,799 | 1,779 |
| 0.615 | 1,251,585 | 41,719 | 39,410 | 3,940 |
| 1.23 | 453,449 | 15,114 | 6,723,039 | 4,652,090 |
| 1.845 | 12,719,605 | 423,986 | 43,295,745 | 29,066,779 |
| 2.768 | 5,738,035 | 191,267 | 23,040,441 | 2,304,044 |
| 3.691 | 1,677,470 | 55,915 | 9,486,444 | 948,644 |

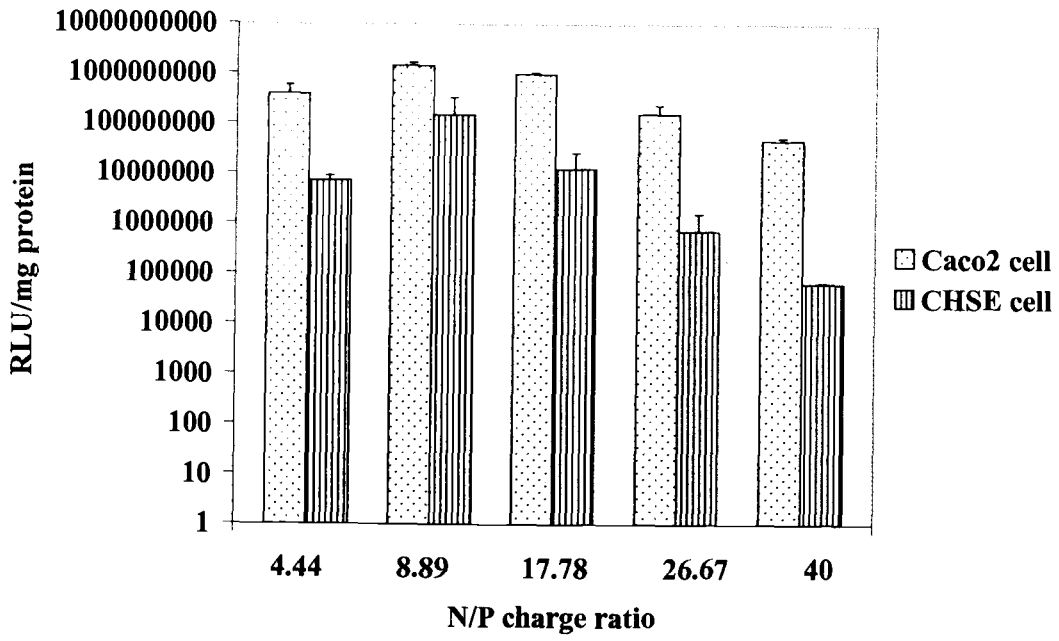



**Figure 3.7: Cell transfected with complexes of PEI and plasmid pCMV-LUC.** Caco-2 cells and CHSE-214 cells were transfected with pCMV-LUC (1.5 μg per well) at 4.44-40 N/P charge ratio. Relative light units (RLU) were normalized to 1 mg of cell protein. Transfection efficiency is maximal at 8.89 N/P charge ratio. Mean ± SD (n=3).

**Table 3.8: Cell transfected with complexes of PEI and plasmid pCMV-LUC**

| N/P charge ratio | RLU/mg protein | | | |
|---|---|---|---|---|
| | Caco-2 cells | SD | CHSE-214 cells | SD |
| 4.44 | 400,975,989 | 194,928,041 | 7,223,391 | 1,788,947 |
| 8.89 | 1,483,034,242 | 203,144,744 | 152,594,528 | 186,191,783 |
| 17.78 | 1,013,700,656 | 70,759,247 | 12,592,647 | 13,916,138 |
| 26.67 | 162,450,514 | 78,991,123 | 719,672 | 869,393 |
| 40 | 47,749,270 | 7,099,844 | 65,441 | 2,181 |

**Table 3.9: Liposome formulations along with optimal amount and carrier to DNA[a] charge ratio for cell transfection**

| Formulation | Ingredients | Amount (μM) | Mole ratio | Total lipid (mg) | Gene expression | Amount (μg) | Charge/ratio |
|---|---|---|---|---|---|---|---|
| 9 | DSPC<br>DOTAP<br>CHOL | 48<br>8<br>32 | 1.5<br>0.25<br>1 | 55.9 | Yes | 30 | 0.96 |
| 10 | DSPC<br>DOTAP<br>CHOL | 32<br>8<br>32 | 1<br>0.25<br>1 | 43.2 | Yes | 25 | 1.028 |
| 11 | DOTAP<br>DOPE | 32<br>32 | 1<br>1 | 46.2 | Yes | 12 | 1.845 |

[a]The amount of DNA used was1.5 μg in all experiments.

**Effect of Vector Type and Lipid Compositions on Gene Transfer to Cells**

At the optimum transfection conditions, PEI provided the best transfection efficiency compared to all liposome formulations. Luciferase expression level of CHSE-214 cells and Caco-2 cells transfected with polyplexes reached $10^8$ and $10^9$ RLU/mg protein and was found to be 2 and 100 fold higher than cells transfected by the complexes of DNA and DOTAP/DOPE, respectively (Figure 3.8).

Among the liposome formulations, liposomes composed of DOTAP/DOPE (liposome formulation 11) gave the best transfection efficiency followed by liposomes composed of (48) DSPC/DOTAP/CHOL and (32) DSPC/DOTAP/CHOL (liposome formulation 9 and 10). Luciferase expression level in Caco-2 cells and CHSE-214 cells transfected with DNA complexed with DOTAP/DOPE was found to be 200-400 fold and 2 fold higher than cells transfected with DNA complexed with (48) DSPC/DOTAP/CHOL and with (32) DSPC/DOTAP/CHOL, respectively (Figure 3.8).

Cells transfected with DNA complexed with PEI and with DOTAP/DOPE demonstrated higher luciferase expression level compared to cells transfected with the complexes of DNA and commercial transfection reagent, (lipofectin®). Luciferase expression level in Caco-2 cells and CHSE-214 cells transfected with polyplexes was found to be 300 fold and 10 fold higher than cells transfected with DNA/lipofectin® complexes, respectively (Figure 3.8). Caco-2 cells and CHSE-214 cells transfected with the complexes of DNA and DOTAP/DOPE showed a 3 fold and 4 fold higher

luciferase expression level compared to cells transfected with DNA/lipofectin® complexes, respectively (Figure 3.8).

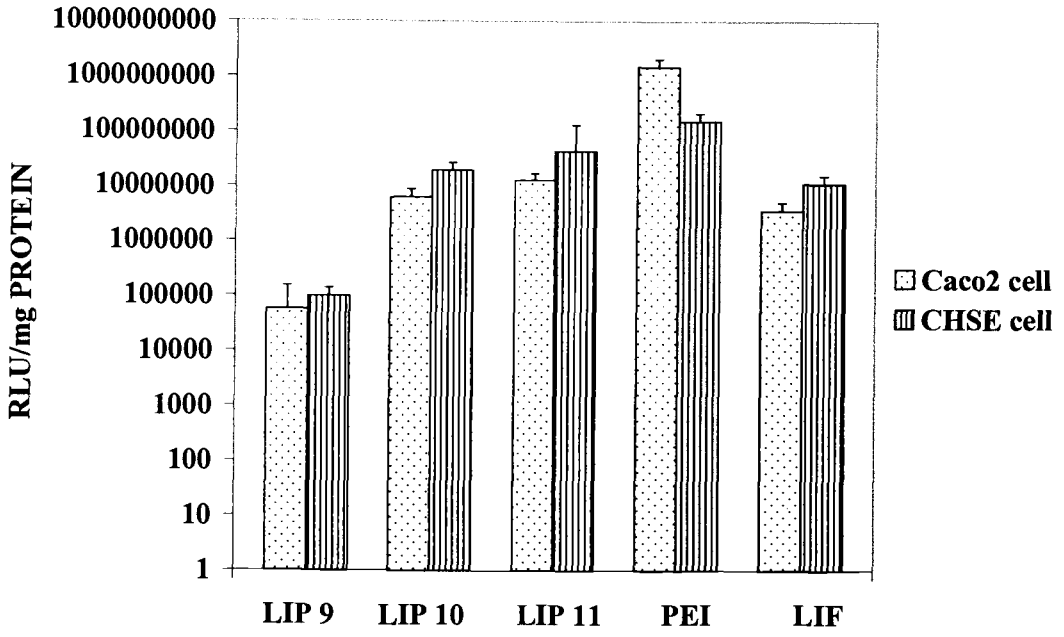



**Figure 3.8: Effect of vector type on luciferase transfer into cells.** Caco-2 cells and CHSE-214 cells were transfected with pCMV-LUC (1.5 μg per well) complexed with different vector formulations. LIP 9, LIP 10, LIP 11, PEI, and LIF represent liposome formulation 9, 10, 11, polyethylenimine, and commercial transfecting reagent (lipofectin®), respectively. Relative light units (RLU) were normalized to 1 mg of cell protein. Transfection efficiency is maximal with PEI. Mean ± SD (n=3).

**Table 3.10: Effect of vector type on luciferase transfer into cells**

| Formulation | RLU/mg protein | | | |
|---|---|---|---|---|
| | Caco-2 cells | SD | CHSE-214 cells | SD |
| LIP9 | 56,903 | 95,065 | 98,676 | 39,470 |
| LIP10 | 6,279,240 | 2,513,216 | 19,287,751 | 7173151 |
| LIP11 | 12,719,605 | 4,231,986 | 43,295,744 | 86,591,489 |
| PEI | 1,483,034,242 | 593,213,697 | 152,594,527 | 61,037,811 |
| LIF | 3,612,820 | 1,445,128 | 11,139,775 | 4,455,909 |

Higher luciferase activity obtained in cells transfected with DNA complexed with DOTAP/DOPE and with PEI was greatly due to their ability to condense plasmid DNA into a small particle, bind to the cells surface via electrostatic interaction, and to protect DNA from DNase degradation (Crook et al., 1996; Godbey et al., 2000). The ability to prevent DNA degradation by DNase was reported to depend on the membrane destabilizing effect of DOPE and PEI. Upon internalization of the DNA complexes into the endosomes, DOPE and PEI fuse with the endosomal membrane resulting in the rupture of endosomal membrane. This process provides the release mechanism for plasmid DNA into the cytosol before reaching cellular lysosomes, subsequently protecting DNA from lysosomal enzymes. Luciferase activity was reduced when DOPE was replaced with DSPC (Guo and Lee, 2000).

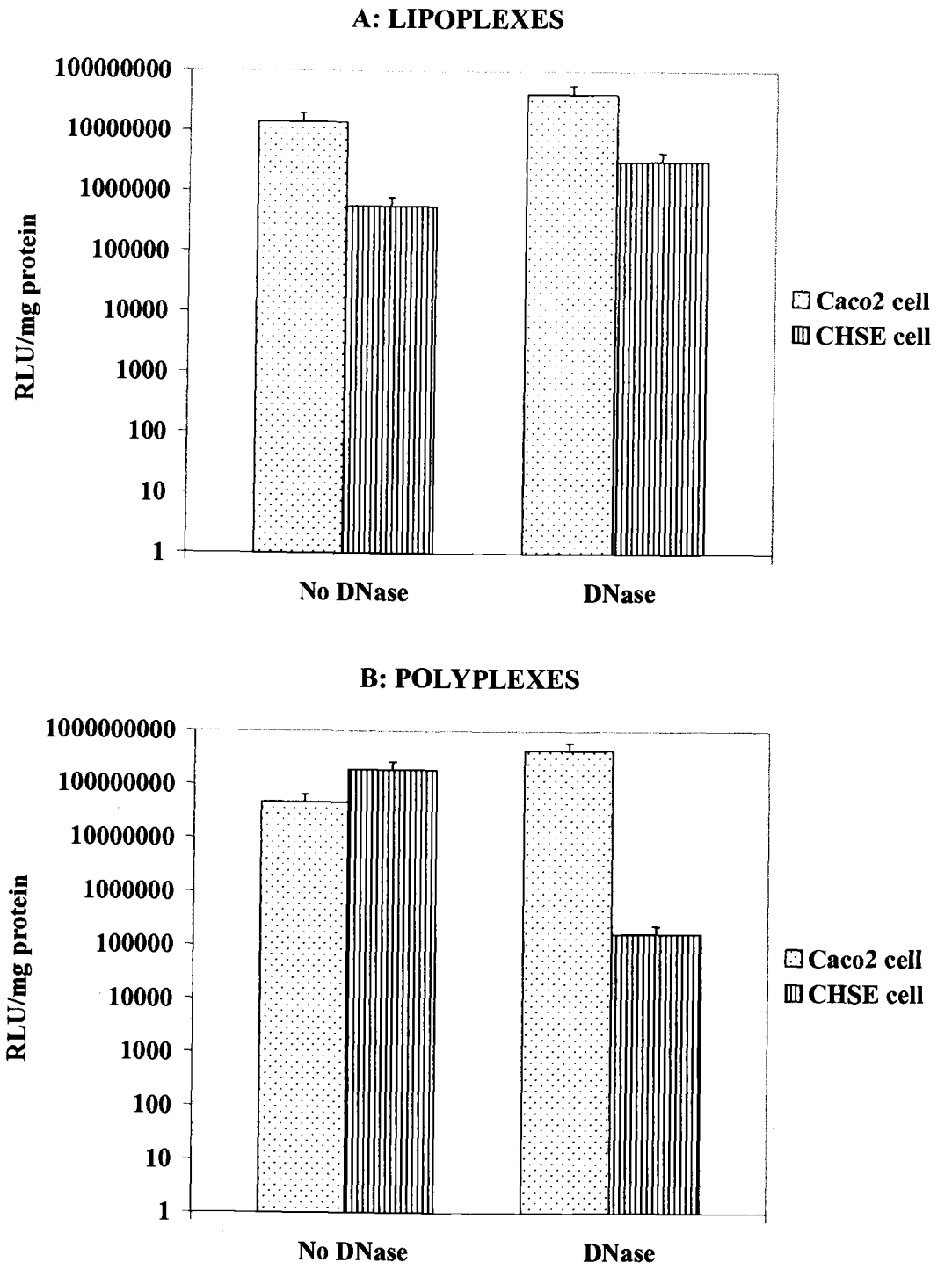
**Stability of DNA against DNase Degradation**

To investigate the protective effect of the cationic liposomes and the cationic polymer on DNA degradation by DNase, complexes of DNA with DOTAP/DOPE and

with PEI were incubated with DNase 1 at 37 °C for 15 minutes in the presence of $MnCl_2/MgCl_2$ alone or $MnCl_2/MgCl_2$ and EDTA prior to application to the cells. $MnCl_2/MgCl_2$ was used to activate DNase 1 activity while EDTA was used to inhibit the function of DNase 1. Cells transfected with either lipoplexes (DNA/DOTAP/DOPE complexes) or with polyplexes (DNA/PEI complexes) in the absence of DNase served as controls.

In the presence of DNase, luciferase expression in Caco-2 and CHSE-214 cells transfected with lipoplexes were approximately 4 fold and 6 fold higher than the cells transfected with lipoplexes in the absence of DNase (control), (Figure 3.9A). The levels of luciferase expression in Caco-2 cells transfected with polyplexes in the presence of DNase were approximately 10 fold higher than the control, (Figure 3.9B). CHSE-214 cells transfected with polyplexes in the presence of DNase showed a 1000 fold lower luciferase expression level compared to the control, (Figure 3.9B). The higher luciferase expression levels observed in Caco-2 cells and CHSE-214 cells treated in the presence of DNase 1 suggests an additional effect of $MnCl_2/MgCl_2$ and EDTA on transfection efficiency of polyplexes and lipoplexes.

**Figure 3.9: Effect of DNase on luciferase expression in cells.** Caco-2 cells and CHSE-214 cells were transfected with (a) pCMV-LUC (1.5 μg per well) complexed with DOTAP/DOPE at 1.845 lipid/DNA charge ratio (b) pCMV-LUC (1.5 μg per well) complexed with PEI (N/P ratio of 8.89) in the presence and absence of DNase 1. Relative light units (RLU) were normalized to 1 mg of cell protein. Mean ± SD (n=3).


A: LIPOPLEXES
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RLU/mg protein
No DNase
DNase
Caco2 cell
CHSE cell
B: POLYPLEXES
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1
RLU/mg protein
No DNase
DNase
Caco2 cell
CHSE cell

**Figure 3.9:**

**Table 3.11: Effect of DNase on luciferase expression in cells transfected with lipoplexes**

| Lipoplexes | RLU/mg protein | | | |
|---|---|---|---|---|
| | Caco-2 cells | SD | CHSE-214 cells | SD |
| No DNase | 13,993,056 | 5,597,222 | 572,341 | 228,936 |
| DNase | 42,172,939 | 16,869,175 | 3,215,616 | 1,286,246 |

**Table 3.12: Effect of DNase on luciferase expression in cells transfected with polyplexes**

| Polyplexes | RLU/mg protein | | | |
|---|---|---|---|---|
| | Caco-2 cells | SD | CHSE-214 cells | SD |
| No DNase | 46,576,497 | 18,630,598 | 189,654,804 | 75,861,921 |
| DNase | 437,364,500 | 174,945,800 | 166,666 | 65,359 |

To evaluate the effect of $MnCl_2/MgCl_2$ and EDTA on transfection efficiency of polyplexes and lipoplexes, cells were transfected with polyplexes and lipoplexes in the presence of $MnCl_2/MgCl_2$ and $MnCl_2/MgCl_2$/EDTA. Caco-2 cells and CHSE-214 cells transfected in the absence of $MnCl_2/MgCl_2$ and EDTA served as a control. In the presence of $MnCl_2/MgCl_2$ alone, luciferase expression in Caco-2 cells and CHSE-214 cells transfected with polyplexes was decreased by 2 fold and 34 fold, respectively, compared to a control, (Figure 3.10). When $MnCl_2/MgCl_2$/EDTA was incorporated into the transfection medium containing polyplexes, the level of expression was increased by 144 fold in Caco-2 cells and 10 fold in CHSE-214 cells compared to a control, (Figure 3.10).

In cells transfected with lipoplexes, the effect of $MnCl_2/MgCl_2$ and EDTA was greater in CHSE-214 cells than in Caco-2 cells. $MnCl_2/MgCl_2$ increased the luciferase expression level in CHSE-214 cells by 6 fold while $MnCl_2/MgCl_2$/EDTA increased the expression level by 10 fold, (Figure 3.11).

Compared to a control, luciferase expression in Caco-2 cells transfected with lipoplexes was increased by double and 4 fold in the presence of $MnCl_2/MgCl_2$ alone and $MnCl_2/MgCl_2$/EDTA, respectively (Figure 3.11).

High expression level observed in the presence of EDTA is possibly due to absorption-enhancing effect of EDTA. Tomita et al., (1996) reported that EDTA enhances paracellular absorption by binding to extracellular calcium resulting in activation of protein kinase C.

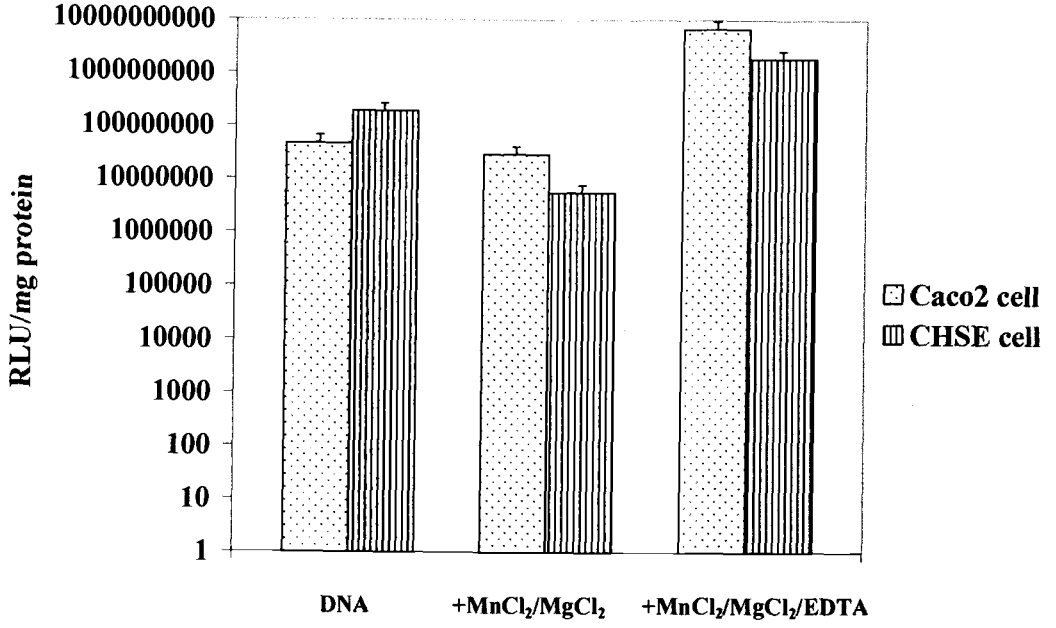


**Figure 3.10: Effect of $MnCl_2/MgCl_2$ and $MnCl_2/MgCl_2$/EDTA on luciferase expression in cells.** Caco-2 cells and CHSE-214 cells were transfected with pCMV-LUC (1.5 μg per well) complexed with PEI (N/P ratio of 8.89) in the absence and presence of $MnCl_2/MgCl_2$ and $MnCl_2/MgCl_2$/EDTA. Cells transfected in the absence of $MnCl_2/MgCl_2$ and EDTA served as a control. Relative light units (RLU) were normalized to 1 mg of cell protein. Mean ± SD (n=3).

**Table 3.13: Effect of $MnCl_2/MgCl_2$ and $MnCl_2/MgCl_2$/EDTA on luciferase expression in cells**

| Ingredients | RLU/mg protein | | | |
|---|---|---|---|---|
| | Caco-2 cells | SD | CHSE-214 cells | SD |
| DNA | 46,576,497 | 18,630,598 | 189,654,804 | 75,861,921 |
| + $MnCl_2/MgCl_2$ | 28,974,583 | 11,589,833 | 5,571,111 | 2,228,444 |
| + $MnCl_2/MgCl_2$/ EDTA | 6,721,788,275 | 2,688,715,310 | 1,866,990,196 | 746,796,078 |

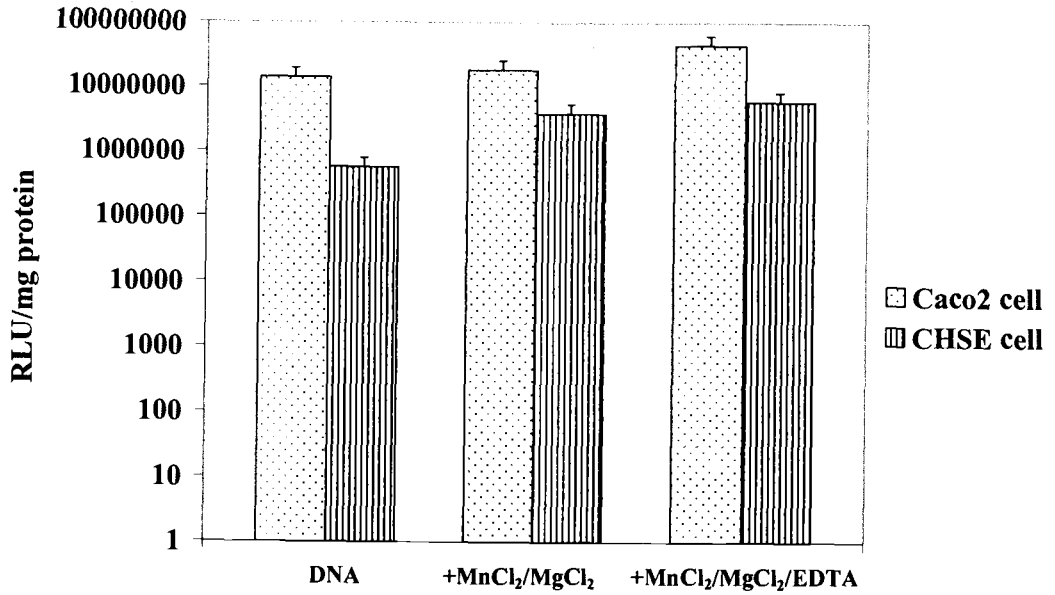


**Figure 3.11: Effect of $MnCl_2/MgCl_2$ and $MnCl_2/MgCl_2$ /EDTA on luciferase expression in cells.** Caco-2 cells and CHSE-214 cells were transfected with pCMV-LUC (1.5 µg per well) complexed with DOTAP/DOPE (lipid/DNA charge ratio of 1.847) in the absence and presence of $MnCl_2/MgCl_2$ and $MnCl_2/MgCl_2$/EDTA. Cells transfected in the absence of $MnCl_2/MgCl_2$ and EDTA served as a control. Relative light units (RLU) were normalized to 1 mg of cell protein. Mean ± SD (n=3).

**Table 3.14: Effect of $MnCl_2/MgCl_2$ and $MnCl_2/MgCl_2$/EDTA on luciferase expression in cells**

| Ingredients | RLU/mg protein | | | |
|---|---|---|---|---|
| | Caco-2 cells | SD | CHSE-214 cells | SD |
| DNA | 13,993,056 | 5,597,222 | 572,341 | 228,936 |
| + $MnCl_2/MgCl_2$ | 18,444,767 | 7,377,906 | 3,915,432 | 1,566,173 |
| +$MnCl_2/MgCl_2$/ EDTA | 44,666,667 | 17,866,666 | 6,072,727 | 2,429,090 |

Further investigations of the effect of $MnCl_2/MgCl_2$ and EDTA on gene transfer into cells are desired to understand more about the mechanism of $MnCl_2/MgCl_2$ and EDTA in cellular uptake of DNA.

To avoid misinterpretation about the protective effect of complexes on DNase degradation, cells transfected with either lipoplexes or polyplexes in the presence of $MnCl_2/MgCl_2$ or $MnCl_2/MgCl_2$/EDTA served as a control for cells transfected with either lipoplexes or polyplexes in the presence of DNase/ $MnCl_2/MgCl_2$ or DNase/ $MnCl_2/MgCl_2$/EDTA, respectively.

The level of luciferase expression in CHSE-214 cells transfected with lipoplexes in the presence of DNase/ $MnCl_2/MgCl_2$ and DNase/ $MnCl_2/MgCl_2$/EDTA was found to be 2.4 fold and 1.9 fold, respectively, lower than the control (Figure 3.12). No difference in luciferase expression was observed from Caco-2 cells transfected with lipoplexes in the presence and absence of DNase (Figure 3.12).

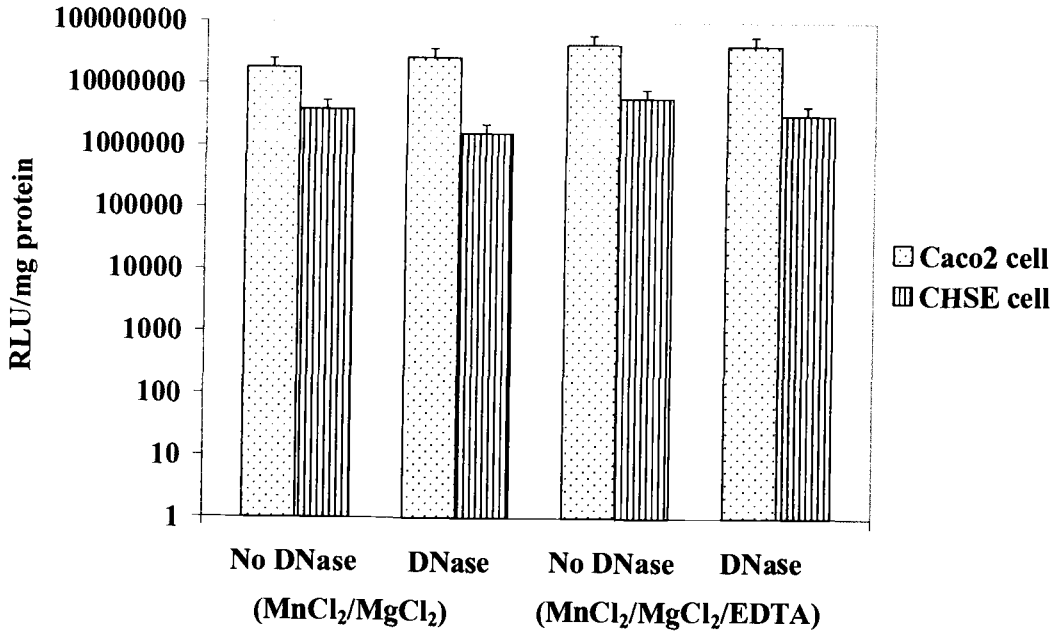


**Figure 3.12: Effect of DNase 1 on luciferase expression in cells transfected with lipoplexes after account for the effect of $MnCl_2/MgCl_2$ and EDTA.** Caco-2 cells and CHSE-214 cells were transfected with pCMV-LUC (1.5 μg per well) complexed with DOTAP/DOPE at 1.845 lipid/DNA charge ratio in the presence and absence of DNase 1. Relative light units (RLU) were normalized to 1 mg of cell protein. Mean ± SD (n=3).

**Table 3.15: Effect of DNase 1 on luciferase expression in cells transfected with lipoplexes after account for the effect of $MnCl_2/MgCl_2$ and EDTA**

| Ingredients | RLU/mg protein | | | |
|---|---|---|---|---|
| | Caco-2 cells | SD | CHSE-214 cells | SD |
| $MnCl_2/MgCl_2$ | 18,444,767 | 7,377,906 | 3,915,432 | 1,566,173 |
| DNase/$MnCl_2/MgCl_2$ | 27,074,030 | 10,829,612 | 1,625,162 | 650,064 |
| $MnCl_2/MgCl_2$/EDTA | 44,666,667 | 17,866,666 | 6,072,727 | 2,429,090 |
| DNase/$MnCl_2/MgCl_2$/ EDTA | 42,172,939 | 16,869,175 | 3,215,616 | 1,286,246 |

Caco-2 cells transfected with polyplexes in the presence of DNase/ $MnCl_2/MgCl_2$ and DNase/ $MnCl_2/MgCl_2$ /EDTA showed an expression level of 1.6 fold and 15 fold, respectively, lower than the control, (Figure 3.13). Luciferase expression level in CHSE-214 cells transfected with polyplexes was decreased by 2 fold in the presence of DNase/ $MnCl_2/MgCl_2$ and by $10^4$ fold in the presence of DNase/ $MnCl_2/MgCl_2$/EDTA compared to the control (Figure 3.13).

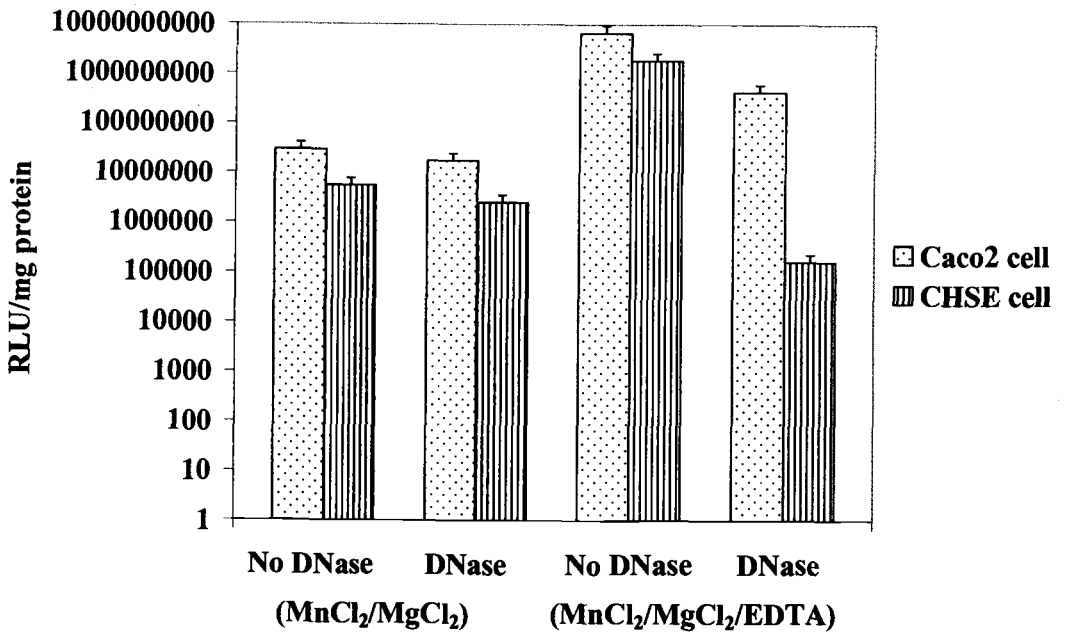


**Figure 3.13: Effect of DNase 1 on luciferase expression in cells transfected with polyplexes after account for the effect of $MnCl_2/MgCl_2$ and EDTA.** Caco-2 cells and CHSE-214 cells were transfected with pCMV-LUC (1.5 μg per well) complexed with PEI at 8.89 N/P charge ratio in the presence and absence of DNase 1. Relative light units (RLU) were normalized to 1 mg of cell protein. Mean ± SD (n=3).

**Table 3.16: Effect of DNase 1 on luciferase expression in cells transfected with polyplexes after account for the effect of $MnCl_2/MgCl_2$ and EDTA**

| Ingredients | RLU/mg protein | | | |
|---|---|---|---|---|
| | Caco-2 cells | SD | CHSE-214 cells | SD |
| $MnCl_2/MgCl_2$ | 28,974,583 | 11,589,833 | 5,571,111 | 2,228,444 |
| DNase/$MnCl_2$/ $MgCl_2$ | 17,695,817 | 7,078,326 | 2,579,610 | 1,031,844 |
| $MnCl_2/MgCl_2$/ EDTA | 6,721,788,275 | 2,688,715,310 | 1,866,990,196 | 746,796,078 |
| Dnase/$MnCl_2$/ $MgCl_2$/EDTA | 437,364,500 | 174,945,800 | 166,666 | 65,359 |

With the exception of cells transfected with polyplexes in the presence of $MnCl_2/MgCl_2$/EDTA, complexation of DNA with DOTAP/DOPE and with PEI apparently protect DNA from DNase degradation. However, lower levels of luciferase expression observed in cells transfected with lipoplexes and with polyplexes in the presence of Dnase 1 indicates that small fraction of DNA was degraded by the enzyme.

A much lower luciferase expression level was observed in cells transfected with polyplexes in the presence of $MnCl_2/MgCl_2$/EDTA was possibly due to the competitive binding of EDTA to PEI. In general, EDTA was added to inhibit DNase activity. However, EDTA possesses an anionic charge that can also interact with cationic charge of PEI resulting in the instability of DNA-complexes and subsequently releasing of DNA from the complexes. The released DNA would be more likely to be subjected to DNase digestion resulting in the reduction of luciferase expression level.

Agarose gel electrophoresis showed that naked DNA was quickly degraded after being exposing to DNase for 2 minutes, (Figure 3.14), while DNA complexed with DOTAP/DOPE and with PEI were still intact after exposure to DNase for at least 30 minutes (Figure 3.15 and 3.16).

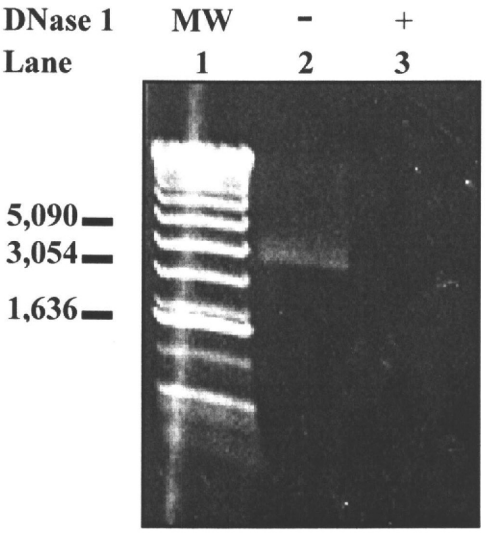


**Figure 3.14: DNase degradation of DNA.** pCMV-LUC was subjected to DNase 1 degradation at 37 °C and run on to 0.6% agarose gel. Each lane was loaded with ~ 0.1 μg of pCMV-LUC. Lane 1 is DNA ladder. Lane 2 is pCMV-LUC in the absence DNase 1. Lane 3 is pCMV-LUV digested with DNase 1 for 2 minutes.

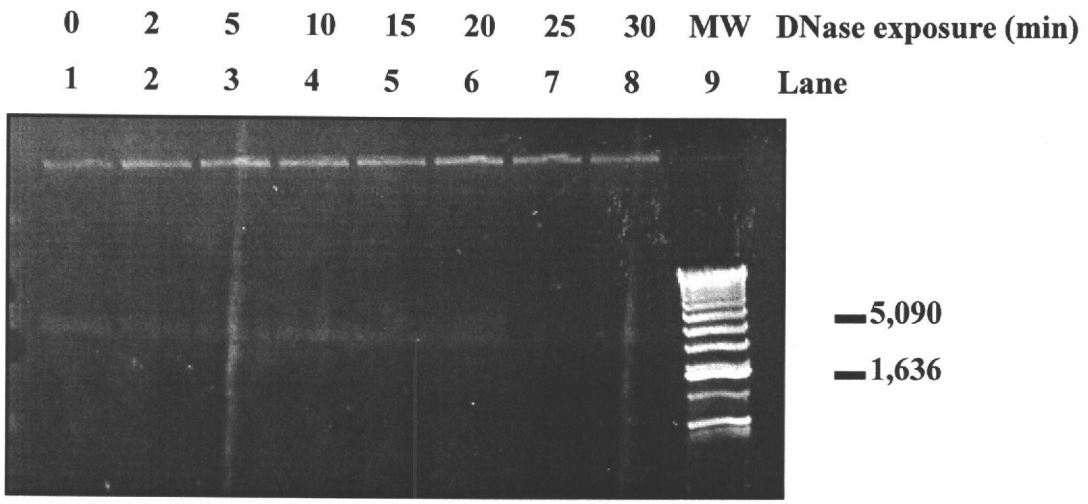


**Figure 3.15: Effect of PEI in protecting DNase degradation of DNA.** pCMV-LUC1 (11.5 μg) was complexed with PEI at 8.89 N/P charge ratio and subjected to DNase degradation with 65 μg of DNase 1 at varying incubation time at 37 °C. The degradation process was stopped by adding 3 μl of 0.5 M EDTA and placing the samples on ice. Heparin was added and the samples were run on 0.6% agarose gel. Each lane was loaded with ~ 0.1 μg of pCMV-LUC. Lane 1-8 is PEI/pCMV-LUC complexes after exposing to DNase 1 for 0, 2, 5, 10, 15, 20, 25, and 30 minutes, respectively. Lane 9 is a DNA ladder.

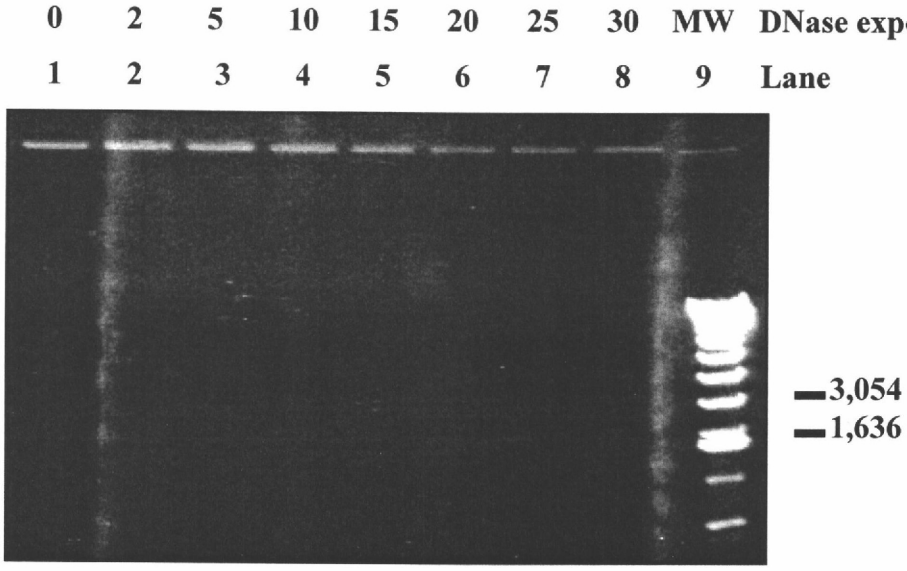


**Figure 3.16: Effect of DOTAP/DOPE in protecting DNase degradation of DNA.** pCMV-LUC1 (11.5 μg) was complexed with DOTAP/DOPE at 1.847 lipid/DNA charge ratio and subjected to DNase degradation with 65 μg of DNase 1 at varying incubation time at 37 °C. The degradation process was stopped by adding 3 μl of 0.5 M EDTA and placing the samples on ice. Heparin was added and the samples were run on 0.6% agarose gel. Each lane was loaded with ~ 0.1 μg of pCMV-LUC. Lane 1-8 is PEI/pCMV-LUC complexes after exposing to DNase 1 for 0, 2, 5, 10, 15, 20, 25, and 30 minutes, respectively. Lane 9 is a DNA ladder.

Once DNA formed complexes with DOTAP/DOPE or with PEI, the interaction of DNA with ethidium bromide was inhibited, thus preventing DNA visualization under a UV lamp (Moret et al., 2001). Heparin, a polyanionic compound, was employed to reduce the stability of DNA-complexes by means of competitive interaction with DNA, subsequently releasing of DNA from the complexes (Moret et al., 2001). The observation of ethidium bromide in the well was more likely due to an

incomplete release of DNA from the complexes, (Figure 3.15 and 3.16). As DNA-complexes are too large to enter the gel, the observation of ethidium bromide in the well suggested that DNA was resistant to DNase digestion but not to ethidium bromide staining (Crook et al., 1996).

This finding was in agreement with the study of Crook et al. (1996), showing no difference in x-gal expression between cells treated with DNA/cationic complexes in the presence and absence of DNase. It was reported that degradation of DNA by DNase can be prevented by complexation of DNA with cationic liposomes (Crook et al., 1996) or cationic polymers (Godbey et al., 2000).

# CONCLUSION

In comparison to the reverse phase evaporation technique, the thin film method allowed the preparation of smaller size liposomes especially when DOTAP and DOPE were incorporated in the formulation. The size of liposomes ranked as DSPC/CHOL > (48) DSPC/DOTAP/CHOL > (32) DSPC/DOTAP/CHOL > DOTAP/DOPE.

Gene expression was observed in both Caco-2 and CHSE-214 cells transfected with DNA/PEI complexes (polyplexes) and with the complexes of DNA and liposomes composed of DOTAP. The level of expression was found to increase with an increase in the ratio of carrier to DNA. At the optimum conditions, positively charged DNA-complexes were found to be more efficiently taken up during transfection compared to less positively or negatively charged complexes; PEI > DOTAP/DOPE > (32) DSPC/DOTAP/CHOL >(48) DSPC/DOTAP/CHOL. The optimum carrier to DNA charge ratio for transfection was found to be 0.96, 1.028, 1.845, and 8.89 for (48) DSPC/DOTAP/CHOL, (32) DSPC/DOTAP/CHOL, DOTAP/DOPE, and PEI respectively. Higher carrier to DNA charge ratio results in the reduction of luciferase expression possibly due to cytotoxicity from high concentration of lipids or polymers as seen by the morphology changes observed in cells (cell shrinkage and detachment from the culture plate). Cells transfected with DNA complexes with PEI and with DOTAP/DOPE demonstrated 10-300 fold and 3-4 fold higher expression level compared to cells transfected with commercial transfection reagent (lipofectin®). Transfection activity was reduced when DOPE was

replaced with DSPC. The DNA complexed with PEI and with DOTAP/DOPE was still intact for at least 30 minutes after exposure to DNase, while non-complexed DNA or naked DNA was degraded by DNase within 2 minutes as determined by agarose gel electrophoresis. Luciferase expression level in Caco-2 and CHSE-214 cells transfected with the complexes of DNA and DOTAP/DOPE indicated no statistical significant difference in luciferase activity between cells treated in the presence and the absence of DNase (p-value > 0.869 for Caco-2 cells and p-value > 0.146 for CHSE-214 cells). Cells transfected with the complexes of DNA and PEI in the presence of DNase show a lower luciferase expression level of $15\text{-}10^4$ fold compared to cells transfected with the complexes of DNA and PEI in the absence of DNase (p-value > 0.016 for Caco-2 cells and p-value > 0.012 for CHSE-214 cells) possibly due to the competitive binding of EDTA to PEI. Cells transfected with polyplexes and lipoplexes in the presence of $MnCl_2/MgCl_2$/EDTA showed higher luciferase expression levels of 10-144 fold and 6-10 fold, respectively compared to cells treated in the absence of $MnCl_2/MgCl_2$/EDTA. These results suggest that DNA transfection efficiency with liposomes and PEI depends on the lipid composition, carrier to DNA charge ratio, and stability of DNA-complexes against DNase. Complexation of DNA with DOTAP/DOPE and with PEI was resistant to DNase degradation, although some portions of DNA in the complexes were digested as determined by cell transfection assay and agarose gel electrophoresis. PEI and liposomes composed of DOTAP/DOPE are efficient vectors in delivering DNA into

both Caco-2 and CHSE-214 cells. Transfection efficiency of PEI and DOTAP/DOPE to deliver DNA into cells can be improved with the use of $MnCl_2/MgCl_2$/EDTA. The most significant finding of this study is an improvement in transfection efficiency of PEI and DOTAP/DOPE in delivering DNA into cells in the presence of $MnCl_2/MgCl_2$/EDTA. Thus, this study could be of significant importance in optimizing and developing oral delivery systems for macromolecules, however more research is needed to understand the mechanism of $MnCl_2/MgCl_2$ and EDTA in cellular uptake of DNA.

ACKNOWLEDGMENT

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# Chapter 4

## *In vivo* Study of Oral Microparticulate DNA Vaccine against Infectious Haematopoietic Necrosis Virus in Rainbow trout

**Angkana Tantituvanont, Benjamin E. Simon, Doug Leisy, and J. Mark Christensen**

## ABSTRACT

Infectious haematopoietic necrosis virus (IHNV) is one of the most hazardous viral diseases in salmon and trout. The financial loss caused by IHNV in the aquaculture industry is significant. Since juvenile fish are more susceptible to the disease the commercial loss usually occurs early. For over two decades, a variety of vaccines have been conceived such as killed, attenuated, subunit IHNV, and DNA vaccines but no licensed product is yet commercially available. In the present work, efforts were made to develop an oral DNA delivery system that will provide effective protection against IHNV. This delivery system was developed by combining together the usefulness of two non-viral vectors, cationic liposomes composed of 1,2-dioleoyl-3-(trimethylammonium)-propane (DOTAP) and dioleoylphosphatidylethanolamine (DOPE), and polycationic polymer, polyethylenimine (PEI), to facilitate membrane uptake of the DNA vaccine and along with enteric-coating the microparticles with Eudragit® L-30 D polymer to prevent DNA degradation in the gastrointestinal tract. Spray drying and spray coating techniques was employed in the microparticle preparation. The objective of this study is to investigate the effectiveness of this oral delivery system by determining the ability of the vaccine to provide protection against IHNV. The efficacy of this oral IHNV vaccine was evaluated by measuring the mortality of vaccinated Rainbow trout fry post waterborne virus challenge. The *in vivo* experiment showed a high mortality (> 80%) in fish vaccinated orally with spray-dried microparticles containing either liposome/DNA complexes or PEI/DNA complexes.

Fish immunized with spray-coated beads demonstrated less mortality (55%). A statistically significant difference in mortality between oral treatment and oral blank treatment (p-value $< 0.002$, chi square test) was observed suggesting an improvement in the level of protection against IHNV when fish were vaccinated with spray-coated beads. The efficacy of the spray-coated oral delivery system in inducing an immune response, however, was not as effective as intramuscular injection of the vaccine. The percent mortality in oral group and injected group was found to be 55.33% and 6%, respectively. Further research is needed in order to develop a safe, effective, and commercially viable vaccine that can control the outbreak of this rhabdovirus.

## INTRODUCTION

The rhabdovirus infectious haematopoietic necrosis virus (IHNV) is one of the most deadly viral diseases of salmon and trout in North America (LaPatra et al., 2001). An epidemic of IHNV may result in losses of 80-100% in juvenile fish, depending upon fish species, virus strain, and environmental conditions (Winton, 1997). When larger fish are infected, a loss of 25% or less is typical. However, the presence of scoliosis in 2-4% of the surviving fish causes them to have a low market value (Winton, 1997). To date, limiting outbreaks of IHNV in fish is performed by destruction of entire infected stocks, the use of virus-free water supplies and using certified non infected eggs for hatching. These practices remain the only method to control outbreaks of IHNV (LaPatra et al., 2001; Winton, 1997). Due to the high loss of fish in the aquaculture industry caused by IHNV, concern for developing an effective and safe vaccine has risen. A considerable need exists to reduce the occurrence, severity, and the inevitable economic cost this rhabdovirus currently produces in the aquaculture industry. For over two decades, a variety of vaccines has been conceived such as killed, attenuated, subunit IHNV, and DNA vaccines, but no licensed product is yet commercially available.

Killed virus vaccines are produced by infecting culture cells with the live virus followed by inactivation of this virus with compounds such as beta-propiolactone (Amend, 1976) and formalin (Nishimura et al., 1985). The post virus challenge mortality of juvenile Rainbow trout vaccinated with a killed virus vaccine was less compared to unvaccinated fish as reported by Amend (1976). The average mortality

was 4% in the vaccinated fish and 70% in the unvaccinated fish (Amend, 1976). An advantage of killed vaccines is the ease of production and license. On the other hand, this vaccine is most efficient when given by injection which is not cost effective for small fish.

Attenuated or live modified virus vaccines are traditionally prepared by serial passage of wild type virus in culture cells until a significant decrease in virulence is observed (Winton, 1997). Fryer et al., (1976) reported that a vaccine of an attenuated strain of IHNV vaccine provided significant protection of Sockeye (*O.nerka*) and Chinook salmon (*O.tshawytscha*) against both injected and waterborne challenge with IHNV (Fryer et al., 1976). Immunization with attenuated vaccines generally provides better humoral and cellular immunity compared to a killed virus because of the replication of the attenuated strain in fish. Attenuated virus strains however have a tendency to revert to virulence, requiring extensive efforts in testing during the development process making initial cost to produce reasonably high. After initial development, the production cost of attenuated vaccines become less expensive and fish vaccination can be easily delivered by the immersion method.

"Subunit vaccines consist of a portion of a pathogen, a single strand protein or important antigenic domain, which will stimulate protective immunity" (Winton, 1997). The study by Engelking and Leong (1989) demonstrated that Kokanee salmon (*O.nerka*) and Rainbow trout fry vaccinated with a subunit vaccine composed of IHNV glycoprotein elicited neutralizing antibodies and protection against lethal virus challenge that approached 100% (Engelking and Leong, 1989). Subunit vaccines are

considered to have a high level of safety due to the fact no infectious virus is present. In the development of IHNV subunit vaccines, bacterial expression systems are commonly used because they have the lowest cost to produce. However, this expression system seems to lack of ability to produce the IHNV glycoprotein in the authentic form, the consequence of this is less efficient protection of fish to the disease.

Despite the safety and high preparation cost of killed and attenuated vaccines and the unreliable efficiency of subunit vaccines, these vaccines have been successful in inducing an immune response against IHNV.

Genetic immunization using naked DNA is one of the newest approaches in vaccine development. This approach has been shown to be effective in inducing an immune response against viral haemorrhagic septicaemia virus (VHSV) (Alonso et al., 2001), infectious haematopoietic necrosis virus (IHNV) (Anderson and Mourich, 1996), peanut allergen (Arah2) (Seder and Gurunathan, 1999). When fish are vaccinated with plasmid DNA, the authentic form of IHNV glycoprotein is produced resulting in an effective long-lasting immune response (Winton, 1997). Presently, vaccination with naked DNA is limited to parenteral injection or gene gun administration. Fish immunization by these methods however is not practical when large numbers of small fish are involved. An alternative approach for vaccine administration such as immersion and the oral route is needed for a more practical delivery system. Oral vaccination provides advantages over injection and immersion vaccination such as no stressful handling, more economical benefit, and relative ease

to administer. The major problem with development of an oral DNA vaccine is the denaturation of plasmid DNA by enzymes and the acidic environment in the stomach and the difficulty in transportation of this plasmid DNA across the cell membrane.

Initial studies from our laboratory demonstrated that non-viral vectors including cationic liposomes composed of DOTAP/DOPE and cationic polymer, polyethylenimine (PEI), are efficient carriers for gene transfer into the cells in both Caco-2 and CHSE-214 cells. In addition, denaturation of plasmid DNA by the stomach environment can be prevented by incorporating the vaccine into enteric-coated microparticles composed of cellulose acetate phthalate (CAP), hydroxypropylmethylcellulose phthalate-55 (HPMCP-55), and Eudragit® L-30 D.

In the present work, an oral DNA vaccine against IHNV was developed by combining the benefits of these non-viral vectors and enteric-coated polymers together. The delivery system was prepared by first forming the complexation of naked DNA with the non-viral vectors followed by incorporating these resulting complexes into enteric-coated microparticles (Figure 4.1) using a spray drying or spray coating technique. This delivery system should provide protection of plasmid DNA against the harsh environment in the stomach, facilitate membrane uptake, and allow the vaccine to reach the site of action and subsequently induce an immune response (Figure 4.2).

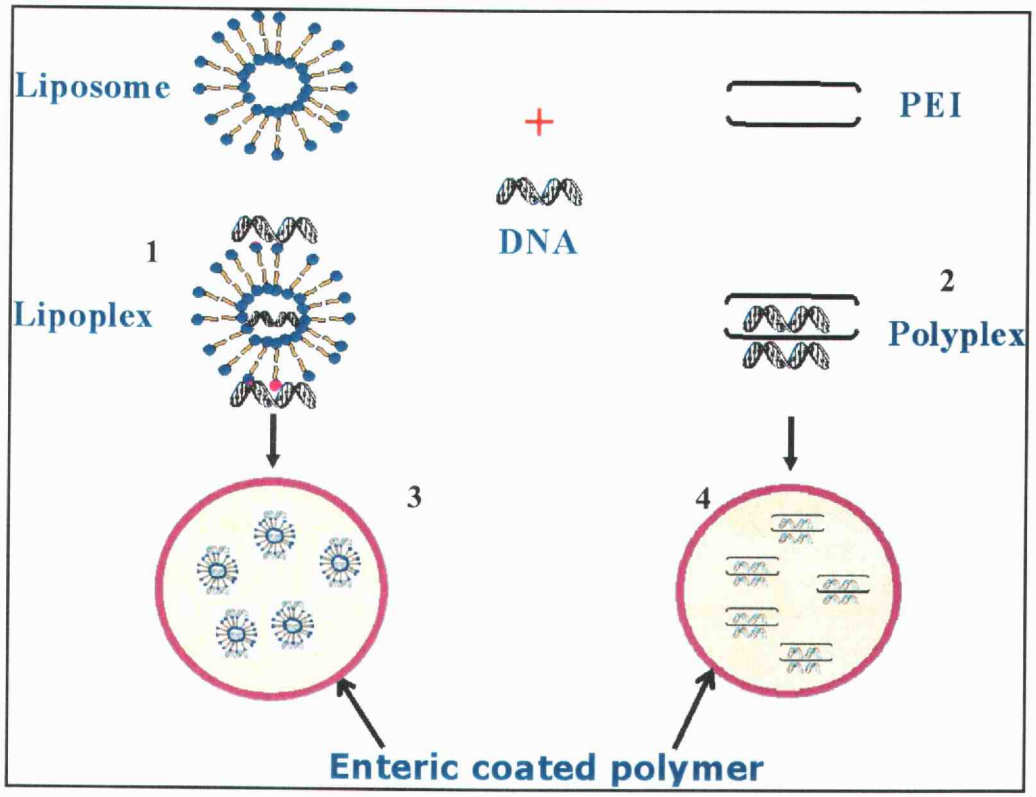



**Figure 4.1: Hypothetical schemes of lipoplex or polyplex embedding in enteric protected polymer.** (1) Complexation of liposome and DNA (lipoplex); (2) Complexation of polyethylenimine (PEI) and DNA (polyplex); (3-4) Incorporation of lipoplex or polyplex in the enteric-coated polymer.

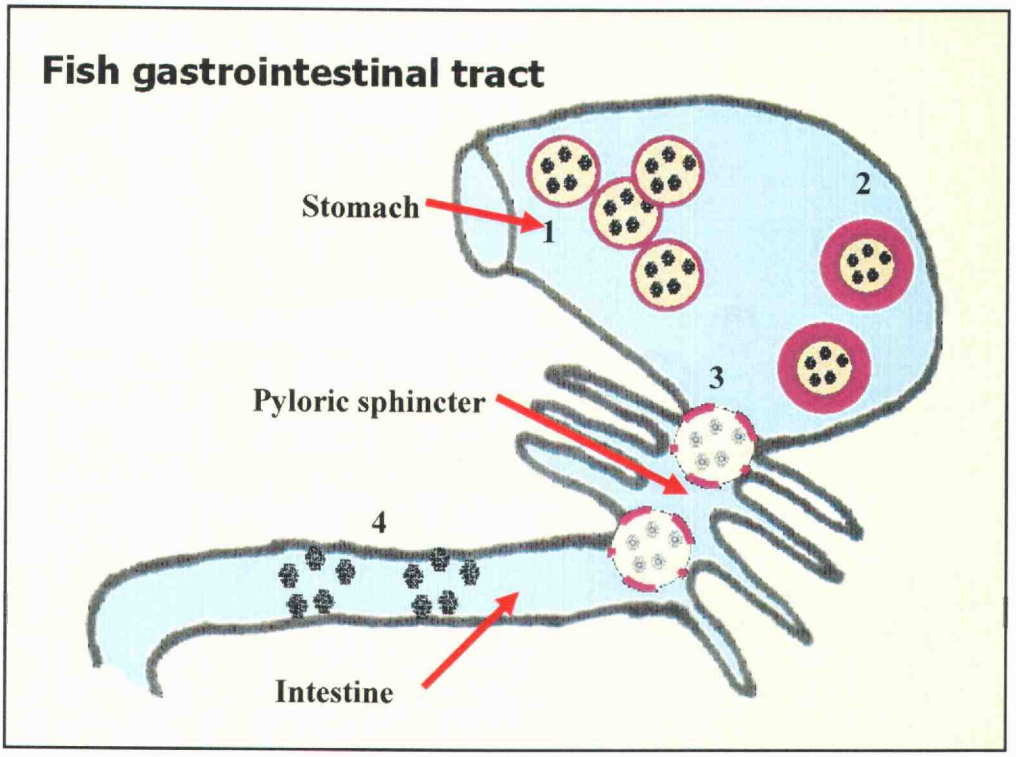



**Figure 4.2a: Hypothetical schemes of enteric protected vaccine after feeding.** (1) Enteric protected microparticles after feeding; (2) Swelling of the enteric protected polymer at the outer surface of particles; (3) Disruption of enteric protected polymer; (4) Release of plasmid DNA into the intestine.

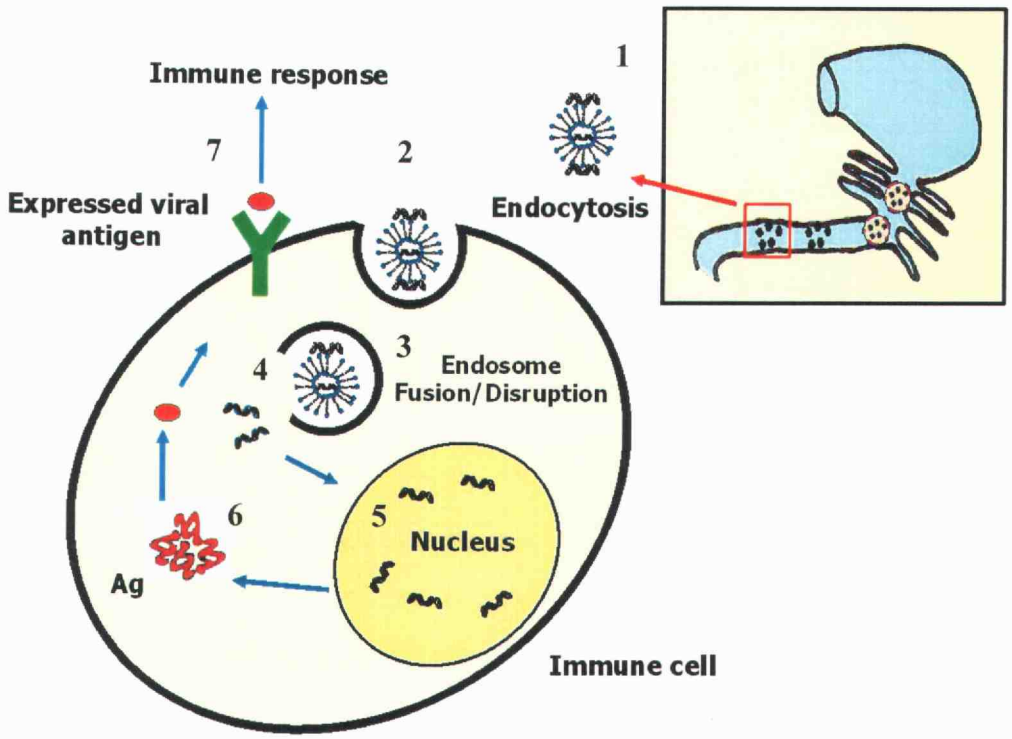



**Figure 4.2b: Hypothetical schemes of gene transfer into eukaryotic cells by cationic liposomes composed of DOPE.** (1) Liposome/DNA complexes enter the eukaryotic cell by endocytosis; (2) The complexes is internalized into endosome; (3) Fusion of liposome to endosomal membrane leading to membrane disruption; (4) Plasmid DNA is released into the cytoplasm of the eukaryotic cell; (5) Entering of plasmid DNA in nucleus; (6) Expression of viral protein antigen; (7) Presenting of viral antigen for stimulating an immune response.

The objective of this study is to investigate the usefulness of this oral delivery system by determining the ability of the vaccine to provide protection against IHNV. The efficacy of the oral IHNV vaccine was evaluated by measuring the mortality of vaccinated rainbow trout fry post waterborne virus challenge.

## MATERIALS AND METHODS

### Polymers and Additives

Eudragit® L-30 D, the acrylic resin polymer from Degussa Rohm Pharma Polymers, Degussa Corporation (Piscataway, NJ, USA) was used as the enteric-coated polymer in the microparticle preparation. Surelease® (ethylcellulose) as a membrane coating was a gift from Colorcon (West Point, PA, USA). Methocel E5 (hydroxypropylmethylcellulose, HPMC-E5) viscosity of 4-6 cps and 4000 cps were purchased from Spectrum, Spectrum Quality Products, Inc. (Gardena, CA, USA). Triethyl citrate, Sigma Chemical Co. (St. Louis, MO, USA) was the plasticizer. Ammonium hydroxide was used for pH adjustment and was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dioleoylphosphatidylethanolamine (DOPE), 1, 2-dioleoyl-3-(trimethylammonium)-propane (DOTAP) were purchased from Avanti Polar-Lipids, Inc. (Alabaster, AL, USA). Polyethylenimine (PEI, 50% w/v) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 3-aminobenzoic acid ethyl ester (MS-222), hepes, Pen-Strep-Fung, gentamycin 10 mg/ml, crystal violet, sodium chloride (NaCl), potassium chloride (KCl), potassium phosphate anhydrous ($KHPO_4$), sodium dihydrate phosphate anhydrous ($NaH_2PO_4 \cdot 7H_2O$), and sodium phosphate dibasic anhydrous ($Na_2HPO_4$) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Formaldehyde was obtained from Fisher Chemical (Fair Lawn, NJ, USA).

**Model Drugs**

Plasmid DNA (pIHNV-G and pCDNA3-Luc) were obtained as a gift from Dr. Joann Leong, Department of Microbiology, Oregon State University (Corvallis, OR, USA). Plasmid DNA encoded with IHNV G-glycoprotein (pIHNV-G) was used to examine immunogenicity in fish while plasmid DNA encoded with firefly luciferase gene (pCDNA3-Luc) was used to examine cell transfection.

**Cell Line**

Chinook salmon (*Oncorhynchus tshawytscha*) embryonic cell line (CHSE-214 cells) was maintained in autoclaved minimum essential media (MEM), Life Technologies Inc. (Rockville, MD, USA) supplemented with 5 % fetal bovine serum (Intergen # 1020-01) and 1% of 200 nM L-Glutamine, Life Technologies Inc. (Rockville, MD, USA). The pH of medium was maintained at 7.4 by buffering with sterile 7.5% sodium bicarbonate solution, Sigma Chemical Co. (St. Louis, MO, USA). CHSE-214 cells were grown and maintained in T 75 $cm^2$ tissue culture flasks at 17 °C.

**Virus**

The type 2 infectious hematopoetic necrosis virus (IHNV), strain 220-90 and strain IHNV-RA, used for lethal challenge was obtained from Benjamin E. Simon and Doug Leisy, Department of Microbiology, Oregon State University (Corvallis, OR, USA).

**Solution Preparation**

Liposome/DNA Complexes and PEI/DNA Complexes Solution

Liposomes, PEI, and plasmid DNA were each diluted with appropriate amounts of solvents as indicated in each formulation (Table 4.1-4.4). The solvents for dilution are sterile deionized water (DI water) for liposomes and 150 mM NaCl solution for PEI. After 15 minutes, plasmid DNA solution was added to either liposomes or PEI solution, with continuous stirring at 500 rpm using a magnetic stirrer to obtain the complexation of DNA and carrier (PEI or liposomes). The resultant liposome/DNA complexes (lipoplexes) or PEI/DNA complexes (polyplexes) solutions were left at room temperature for 30-45 minutes before further use.

Eudragit® L-30 D Solution for Enteric-Coated Spray-Dried Microparticle Preparation

Fifty three grams of Eudragit® L-30 D suspension (30% w/v) containing 16 g of solid polymer was diluted with 50 ml of sterile DI water and magnetically stirred at 100 rpm for 15 minutes. Plasticizer (TEC) in the amount of 1.6 g was added into polymer suspension followed by another 15 minutes of stirring. The polymer suspension was dissolved by adjusting the pH of suspension to 7.4 with ammonium hydroxide (Table 4.1-4.5). The resultant solution was mixed thoroughly for 20 minutes using a magnetic stirrer.

Eudragit® L-30 D Coating Solution for Enteric-Coated Beads Preparation

One hundred grams of Eudragit® L-30 D suspension (30% w/v) containing 30 g solid polymer was diluted with 30 ml of sterile deionized water and stirred on a magnetic stirrer at 100 rpm for 15 minutes. Plasticizer (TEC) in the amount of 7.5 g was added to the polymer suspension followed by an additional of 15 minutes stirring. Talc (1.5 g) was added gradually to the polymer suspension containing plasticizer with continuous stirring at 100 rpm for 30 minutes (Table 4.7-4.8). The resultant polymer suspension was then filtered through a 20 mesh screen to remove the clumps and to maintain the homogeneity of the polymer suspension.

HPMC E-5 Coating Solution for Enteric-Coated Beads Preparation

One gram of HPMC E-5 powder was dispersed thoroughly into 105 ml of hot deionized water in 250 ml bottle. The obtained dispersion was sterilized by autoclaving for 30 minutes. The sterile solution was immediately placed in a temperature controlled room at 4 °C and the sterile solution was continuously stirred at 300 rpm until a clear solution was observed. Due to evaporation during the process of autoclaving, the final volume of solution was approximately 100 ml. The resultant solution was used as a stock solution (10 mg/ml).

Surelease® Coating Solution for Enteric-Coated Beads Preparation

Ethylcellulose (Surelease®), coating solution was simply prepared by mixing a 1:1 ratio of surelease® solution (25.4% w/v solid) and sterile deionized water with continuous stirring at 100 rpm for 20 minutes.

Methyl Cellulose Overlay Media

Methyl cellulose solution was prepared by dispersing 1.5 g of methyl cellulose (4000 cps) into 165 ml of boiling deionized water 200 ml bottle. The dispersion was shaken rigorously and autoclave immediately for 30 minutes. The sterile solution was immediately placed in the temperature controlled room at 4 °C and the sterile solution was continuing stir at 300 rpm until a clear solution was observed. Due to evaporation during the process of autoclaving, the final volume of solution was approximately 158 ml. Methyl cellulose overlay media was prepared by adding the following ingredients into 158 ml of cooled methyl cellulose solution.

| | | |
|---|---|---|
| 10X MEM | 20 | ml |
| Hepes 280 mM | 10 | ml |
| Pen-Strep-Fung | 2 | ml |
| Gentamycin 10 mg/ml | 1 | ml |
| L-Glutamine 100X | 2 | ml |
| Fetal bovine serum | 10 | ml |

Crystal Violet Formalin Stain Solution

| | | |
|---|---|---|
| Crystal violet | 0.5 | g |
| Deionized water | 450 | ml |
| 37% formaldehyde (Formalin) | 50 | ml |

Phosphate Buffered Saline (PBS) pH 7.4

| | | |
|---|---|---|
| 137 mM NaCl | 8.0 | g |
| 2.7 mM KCl | 0.2 | g |
| 4.3 mM $NaHPO_3 \cdot 7H_2O$ | 1.15 | g |
| 1.4 mM $KHPO_4$ | 0.2 | g |
| Deionized water | 990 | ml |

10% Neutral Buffered Formalin (pH 7.0-7.4)

| | | |
|---|---|---|
| Formalin | 100 | ml |
| 33 mM $NaH_2PO_4 \cdot H_2O$ | 4.6 | g |
| 46 mM $Na_2HPO_4$ | 6.5 | g |
| Deionized water | 889 | ml |

## Preparation of Experimental Treatments

Enteric-Coated Spray-Dried Microparticle Treatment

Microparticles embedded with liposome/DNA complexes (lipoplexes) or PEI/DNA complexes (polyplexes) were produced using a spray drying technique. The spray solution was prepared according to the formulation listed in Table 4.1-4.4. Briefly, Lipoplex or polyplex solutions were added separately into Eudragit® L-30 D solution (16 mg/ml) with continuous stirring at 100 rpm using a magnetic stirrer for later spray drying. The obtained polymer solution was atomized through a 0.5mm spray nozzle with a constant feed rate of 30 ml/hr from the peristaltic pump, Rainin Instrument Co., Inc. (Woburn, MA, USA). Atomized air pressure of 15 psi was set along with 50% of full air capacity from the fluidized bed unit. The inlet and outlet drying temperatures were set at 60 °C and 40 °C, respectively. The spray drying conditions are presented in Table 4.6.

**Table 4.1: Formulation of spray-dried microparticles for oral pIHNV-G vaccine using DOTAP/DOPE as carrier vector (Treatment 1)**

| Type of solution | Ingredients | Amount |
|---|---|---|
| DNA solution | pIHNV-G (mg) | 8 |
| | DI. water (dilution solvent, ml) | 80 |
| Liposome solution | DOTAP/DOPE (1 mg/ml) | 64 |
| | DI. water (dilution solvent, ml) | 80 |
| Eudragit® L-30 D solution | Eudragit® L-30 D (30% w/v) (g) | 53.34 (Total solid 16 g) |
| | Triethyl Citrate (TEC, g) | 1.6 |
| | $NH_4OH$ adjust pH to 7.4 | |
| | Sterile DI. water (ml) | 50 |

**Table 4.2: Formulation of spray-dried microparticles for oral pCDNA3-LUC vaccine using DOTAP/DOPE as carrier vector (Treatment 2)**

| Type of solution | Ingredients | Amount |
|---|---|---|
| DNA solution | pCDNA3-LUC (mg) | 8 |
| | DI. water (dilution solvent, ml) | 80 |
| Liposome solution | DOTAP/DOPE (1 mg/ml) | 64 ml |
| | DI. water (dilution solvent, ml) | 80 |
| Eudragit® L-30 D solution | Eudragit® L-30 D (30% w/v) (g) | 53.34 (Total solid 16 g) |
| | Triethyl Citrate (TEC, g) | 1.6 |
| | $NH_4OH$ adjust pH to 7.4 | |
| | Sterile DI. water (ml) | 50 |

**Table 4.3: Formulation of spray-dried microparticles for oral pIHNV-G vaccine using PEI as carrier vector (Treatment 3)**

| Type of solution | Ingredients | Amount |
|---|---|---|
| DNA solution | pIHNV-G (mg) | 8 |
| | DI. water (dilution solvent, ml) | 80 |
| PEI solution | PEI (0.9 mg/ml) | 22 ml |
| | DI. water (dilution solvent, ml) | 80 |
| Eudragit® L-30 D solution | Eudragit® L-30 D (30% w/v) (g) | 53.34 (Total solid 16 g) |
| | Triethyl Citrate (TEC, g) | 1.6 |
| | $NH_4OH$ adjust pH to 7.4 | |
| | Sterile DI. water (ml) | 50 |

**Table 4.4: Formulation of spray-dried microparticles for oral pCDNA3-LUC vaccine using PEI as carrier vector (Treatment 4)**

| Type of solution | Ingredients | Amount |
|---|---|---|
| DNA solution | pCDNA3-LUC (mg) | 8 |
| | DI. water (dilution solvent, ml) | 80 |
| PEI solution | PEI (0.9 mg/ml) | 22 ml |
| | DI. water (dilution solvent, ml) | 80 |
| Eudragit® L-30 D solution | Eudragit® L-30 D (30% w/v) (g) | 53.34 (Total solid 16 g) |
| | Triethyl Citrate (TEC, g) | 1.6 |
| | $NH_4OH$ adjust pH to 7.4 | |
| | Sterile DI. water (ml) | 50 |

**Table 4.5: Formulation of spray-dried microparticles for oral blank treatment (Treatment 5)**

| Type of solution | Ingredients | Amount |
|---|---|---|
| Eudragit® L-30 D solution | Eudragit® L-30 D (30% w/v) (g) | 53.34 (Total solid 16 g) |
| | Triethyl Citrate (TEC, g) | 1.6 |
| | $NH_4OH$ adjust pH to 7.4 | |
| | Sterile DI. water (ml) | 50 |

**Table 4.6: Operating conditions for spray-dried microparticle preparation**

| Factors | Level |
|---|---|
| Nozzle size (mm) | 0.5 |
| Flow rate (ml/hr) | 30 |
| Nozzle Air (Psi) | 15 |
| Fluidize Air Pressure | 50% of full capacity |
| Inlet-Outlet Drying Air Temperature (°C) | 60-40 |

Enteric-Coated Non-Pariel Sugar Beads Treatment

Enteric-coated oral DNA vaccines were prepared by multiple layer coating of non-pariel sugar beads using spray coating technique. Spray coating machine was Niro Fluid Bed Laboratory Unit STEREA-1, Niro Inc., Aeromatic-Fielder Div. (Columbia, MD, USA). One hundred grams of non-pariel sugar beads of the size range of 40-50 mesh were first coated with 1% ethylcellulose (Surelease®) coating solution to improve bead hardness. HPMC E-5 solution in the amount of 0.015% was employed as a second coat to increase the surface adhesiveness. The PEI/DNA complex (polyplexes) solution was applied as a third coat followed lastly by a coat of Eudragit® L-30 D coating solution to form an enteric-coated layer. Note, that for the preparation of blank enteric-coated beads, the third coat contained only the PEI solution. The formulation of enteric-coated beads oral pIHNV-G vaccine and enteric-coated oral blank treatment are listed in Table 4.7 and 4.8, respectively. The spray-

coating conditions for HPMC-E5, ethylcellulose (Surelease®), PEI/DNA complexes, and Eudragit® L-30 D coating solution is presented in Table 4.9-4.12.

**Table 4.7: Formulation of enteric-coated beads[a] for oral pIHNV-G vaccine (Treatment 8)**

| Coating solution | Ingredients | Amount |
|---|---|---|
| Surelease® solution | Surelease® | 1 g solid |
| | Sterile deionized water | 4 ml |
| HPMC E-5 | HPMC-E5 (10 mg/ml) | 15 |
| | | (1.5 ml of stock solution) |
| | Deionized water (ml) | 20 |
| PEI solution | PEI (0.9 mg/ml) | 20 ml |
| | Sterile 150 mM NaCl solution | 100 ml |
| DNA solution | pIHNV-G | 5 mg |
| | Sterile 150 mM NaCl solution | 125ml |
| Eudragit® L-30D | Eudragit® L-30D (30% w/v) | 40 g solid |
| | TEC | 10 g |
| | | (25% of solid polymer) |
| | Talc | 2 g |
| | | (5% of solid polymer) |
| | Sterile deionized water | 30 g |
| | $NH_4OH$ adjust pH to 7.4 | |

[a]The amount of non-pariel sugar beads (40-50 mesh) was 100 gram

**Table 4.8: Formulation of enteric-coated beads[a] for oral blank treatment (Treatment 9)**

| Coating solution | Ingredients | Amount |
|---|---|---|
| Surelease® solution | Surelease® | 1 g solid |
| | Sterile deionized water | 4 ml |
| HPMC E-5 | HPMC-E5 (mg) | 15 |
| | | (1.5 ml of stock solution) |
| | Deionized water (ml) | 20 |
| PEI/DNA complexes | PEI (0.9 mg/ml) | 20 ml |
| | Plasmid DNA (mg) | - |
| | 150 mM Sterile | 225 |
| | NaCl solution (ml) | |
| Eudragit® L-30 D | Eudragit® L-30 D | 40 g solid |
| | TEC | 10 g |
| | | (25% of solid polymer) |
| | Talc | 2 g |
| | | (5% of solid polymer) |
| | Sterile deionized water | 40 g |
| | $NH_4OH$ adjust pH to 7.4 | |

[a]The amount of non-pariel sugar beads (40-50 mesh) was 100 gram

**Table 4.9: HPMC E-5 solution coating conditions**

| Factor | Level |
|---|---|
| Nozzle (mm) | 0.5 |
| Inlet and outlet temperature (°C) | 60-50 |
| Atomized air pressure (psi) | 10 |
| Fluidized air | 50% of full capacity |
| Solution feed rate (ml/min) | 1-1.5 |

**Table 4.10: Ethylcellulose (Surelease®) solution coating conditions**

| Factor | Level |
|---|---|
| Nozzle (mm) | 0.5 |
| Inlet and outlet temperature (°C) | 60-47 |
| Atomized air pressure (psi) | 10-15 |
| Fluidized air | 70% of full capacity |
| Solution feed rate (ml/min) | 1-1.5 |

**Table 4.11: PEI/DNA complexes solution coating conditions**

| Factor | Level |
|---|---|
| Nozzle (mm) | 0.5 |
| Inlet and outlet temperature (°C) | 50-40 |
| Atomized air pressure (psi) | 10 |
| Fluidized air | 50%-70% of full capacity |
| Solution feed rate (ml/min) | 0.8-1.5 |

**Table 4.12: Eudragit® L-30 D solution coating conditions**

| Factor | Level |
|---|---|
| Nozzle (mm) | 0.5 |
| Inlet and outlet temperature (°C) | 50-40 |
| Atomized air pressure (psi) | 10-15 |
| Fluidized air | 50% of full capacity |
| Solution feed rate (ml/min) | 2-3 |

### *In vitro* Release of pIHNV-G from Enteric-coated Beads

The *in vitro* release studies of pIHNV-G entrapped in the enteric-coated beads were performed in triplicate. Briefly, 0.4 g of enteric-coated beads loaded with pIHNV-G were incubated in 5 ml of sterile simulated gastric fluid pH 1.23 for 3 hours followed by 1 hour in sterile simulated intestinal fluid pH 7.4. Samples of 4 ml were removed at 10, 15, 30, 60, 90, 120, 180, 185, 190, 195, 200, 210, 225, 240 minutes. The amount of pIHNV-G release from the enteric-coated beads was determined by measuring the absorbance of the solution at the wavelength of 260 nm using a spectrophotometer, Beckman DU-600, Beckman Coulter, Inc. (Fullerton, CA, USA).

### Analysis of Structural and Functional Integrity of Plasmid DNA

Structural and functional integrity of extracted plasmid DNA from enteric-coated spray-dried microparticles was determined by agarose gel electrophoresis and transfection assay with CHSE-214 cells.

Agarose Gel Electrophoresis

Thirty milligrams of spray-dried microparticles corresponding to 1-1.5 μg, pCDNA3-LUC were dissolved first with sterile phosphate buffer (pH 7) followed by DNA purification using QIAquick PCR purification kit. The extracted plasmid was mixed with 5 μl gel loading buffer (bromophenol blue 0.05% w/v, sodium dodecyl sulphate 0.05% w/v, sucrose 40% w/v EDTA 0.1 M pH 8) and subjected to agarose gel electrophoresis in the presence of ethidium bromide for 10 minutes at 210 Volts. DNA visualization of the gels was carried out under UV lamp, Fotodyne (New Berlin, WA, USA).

Cell Transfection

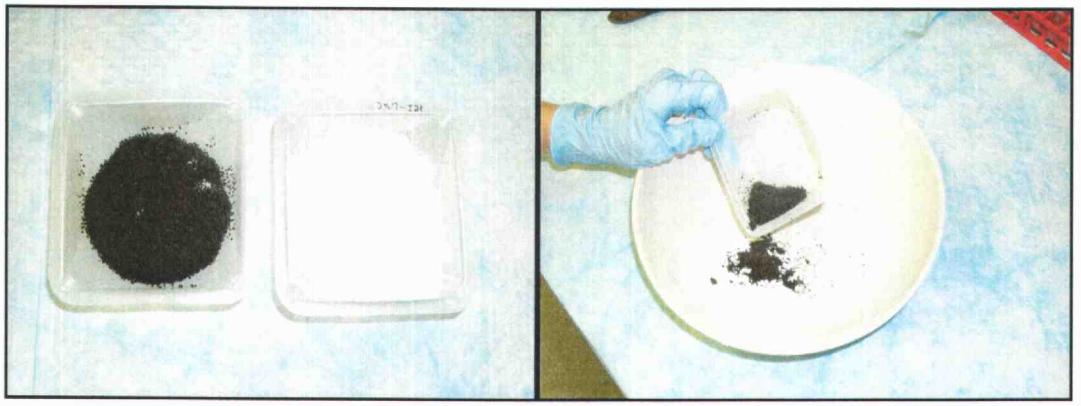
CHSE-214 cells were seeded in 12 well culture plates at a cell density of $1 \times 10^6$ cells per well 24 hours before transfection. Cells were allowed to attach to the plate surface as a monolayer by growing in MEM supplemented with 5% FBS, at 17 °C. Prior to transfection, cells with 60-70% confluence were washed twice with 1 ml serum free medium (MEM supplemented with 1% L-Glutamine) and then covered with 0.8 ml of serum free medium before adding transfection complexes (Complexes of DNA and lipofectin®). Approximately 0.2 ml of transfection complexes were added gently to cells in each well. Cells were incubated for 3 hours at 17 °C. After incubation for 3 hours, the transfection medium was removed and replaced with 1 ml complete medium (MEM supplemented with with 5 % fetal bovine serum and 1% of

L-Glutamine), and cells were further cultured for at least 72 hours. Transfection was performed in triplicate with lipofectin® complexed with extracted pDNA3-LUC and with control pCDNA3-LUC. Cells treated with DNA/lipofectin® complexes and with naked DNA served as a positive control and negative control, respectively. Transfection complexes were prepared according to the Promega's instruction for lipofectin®. The amount of DNA used was ~ 1μg in all test experiments. To 1 μg of DNA 10 μl of lipofectin® reagent was needed.

**Granulation of Treatment Feed**

Granulation of Enteric-Coated Spray-Dried Microparticles For Vaccine Treatment

The granule pellets of each treatment group (Treatment 1 through 5) were prepared by granulating 1:1:0.25 mixture of the enteric-coated spray-dried microparticles to the regular feed, (Starter # 1 trout pellets, Life stage diet for fish, Bio-Oregon) to corn starch with fish oil. The granule was obtained by extruding the feeding mass through 1 mm screen (Figure 4.3). The final granule was then kept in the desiccators at -20 °C.



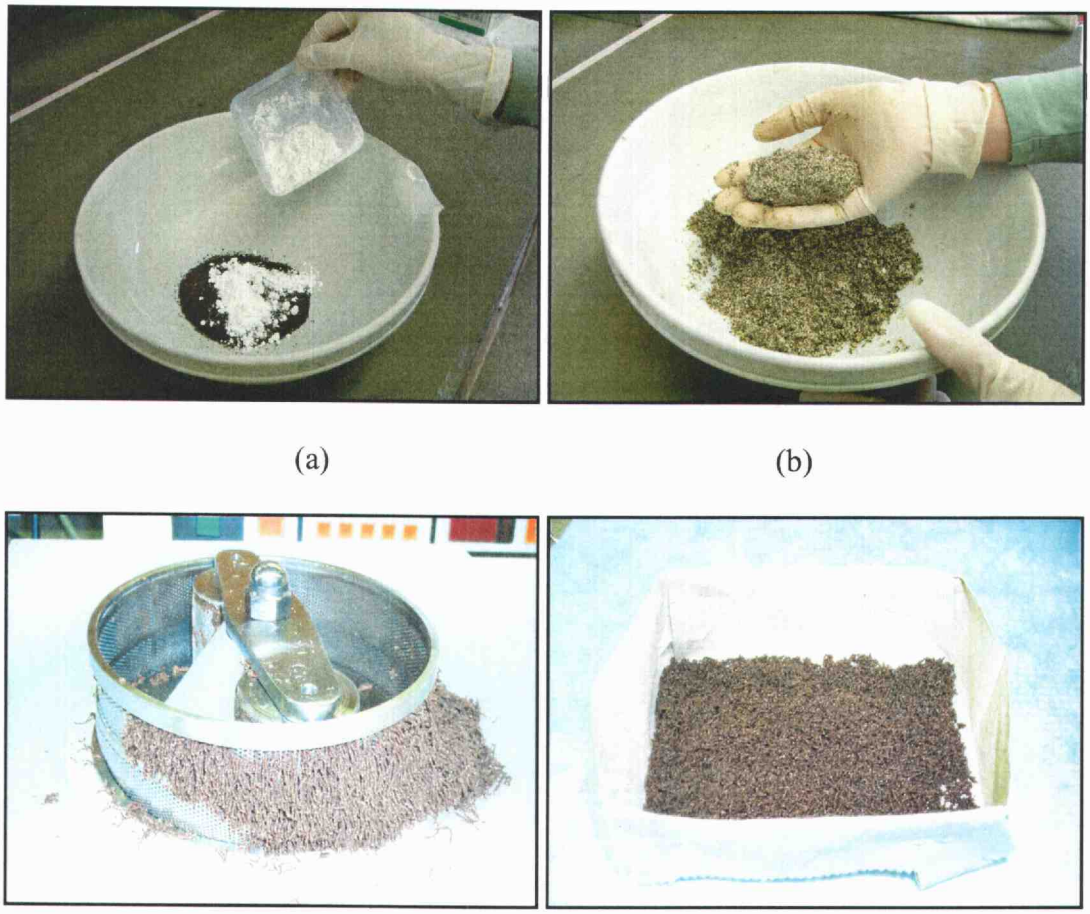
(a) (b)

(c) (d)

**Figure 4.3: Granulation of enteric-coated spray-dried microparticles vaccine.**
(a) 1:1 ratio of enteric-coated spray-dried microparticles and autoclaved fish feed.
(b) The mixture of enteric-coated spray-dried microparticles and autoclaved feed.
(c) Extrusion of feed mass through 1 mm screen.
(d) Granule treatment feed.

Granulation of Enteric-Coated Non-Pariel Sugar Beads Vaccine Treatment

The treatment granules were prepared by granulating 2:1:0.5 the mixture of DNA enteric-coated beads to regular fish feed, (Starter # 1 trout pellets, Life stage diet for fish, Bio-Oregon) to corn starch. Sterile deionized water pH 4 was used as a binding solvent. The granule was obtained by extruding the feed mass through a 1 mm screen using an extruder, CALEVA process LTD, Dorset England (Figure 4.4). The resultant vaccine feed granule was dried overnight in the oven at 37 °C. Afterwards, the dried granule vaccine feed was kept in the tight container at room temperature.



(a) (b)

(c) (d)

**Figure 4.4: Granulation of enteric-coated non-pariel sugar beads vaccine.**
(a) 2:1 ratio of regular fish feed and corn starch.
(b) Feed mass containing enteric-coated beads treatment.
(c) Extrusion of feed mass through 1 mm screen.
(d) Granule treatment feed.

## *In vivo* Experiment

### Experimental Animals

Rainbow trout's eggs were bought from Rainbow Trout Lodge (Sumner, WA, USA) and hatched (Figure 4.5) at the salmon disease laboratory, Oregon State University (Corvallis, OR, USA). Fish were supplied with 13 °C specific pathogen free well water and fed daily with trout pellets (starter # 1, Life stage diets for fish, Bio-Oregon) in 100 liter tanks.

### Experimental Treatments

Enteric-Coated Spray-Dried Microparticles Vaccine Treatment

The Seven vaccine treatments for the fish trial experiment were:

1. Enteric-coated oral liposome/pCDNA-LUC spray-dried microparticles vaccine.
2. Enteric-coated oral liposome/ pIHNV-G spray-dried microparticles vaccine.
3. Enteric-coated oral PEI/pCDNA3-LUC spray-dried microparticles vaccine.
4. Enteric-coated oral PEI/ pIHNV-G spray-dried microparticles vaccine.
5. Enteric-coated oral Eudragit® L-30 D spray-dried microparticles vaccine
6. pCDNA3-LUC intramuscular injection.

7. pIHNV-G intramuscular injection.

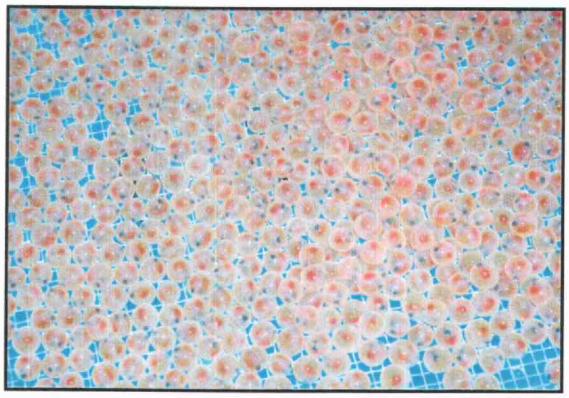
Treatment 7 served as a positive control in lethal challenge experiment while treatment 6 served as a positive control from the cell transfection experiment. Treatment 5 served as a negative control in both lethal challenge and cell transfection experiment.

Enteric-Coated Non-Pariel Sugar Beads Vaccine Treatment

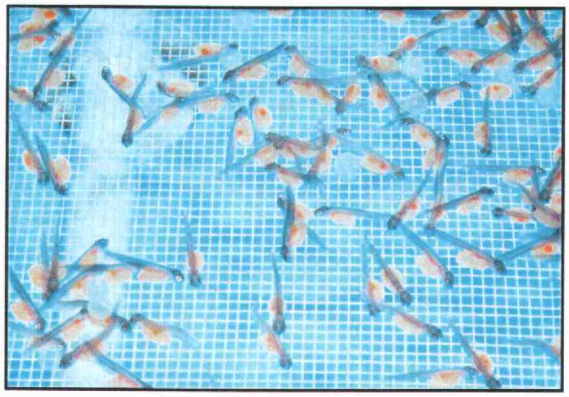
The four treatments for the fish trial experiment were

8. Enteric-coated Oral PEI/pIHNV-G non-pariel sugar beads vaccine.
9. Enteric-coated Oral PEI non-pariel sugar beads vaccine
10. pIHNV-G intramuscular injection
11. PBS intramuscular injection

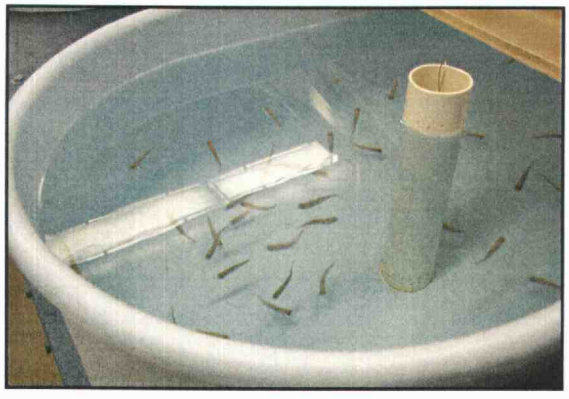
Treatment 10 served as a positive control for the lethal challenge experiment. Treatment 9 and 11 served as negative controls for the oral group and injection group, respectively.



(a)



(b)



(c)

**Figure 4.5: The life cycle of Rainbow trout.** Panel A shows an embryonic stage (eggs); Panel B shows a larval stage; Panel C shows a juvenile Rainbow trout fry.

## Immunization experiment

*Immunization with Enteric-Coated Spray-Dried Microparticles DNA Vaccine*

Fish Distribution

Two hundred and ten fish were randomly distributed into 5×100 liter tanks for the oral treatment groups, while eighty seven fish were randomly assigned to 2×100 liter tanks for the intramuscular (IM) injection treatment groups. Tanks were randomly distributed in the region and assigned numbers 1-7 (Figure 4.6). Fish were distributed into each tank by randomly drawing cards and by using the card order placing 25 fish into the tank until all tanks had 25 fish. The cards were then shuffled and another 25 fish were assigned again into each tank by order of the cards drawn. The process was repeated until the numbers of fish in each tank reached 210 for oral treated groups and 87 for injected group.

| A1 | A2 | A3 | A4 | A5 | A6 | A7 |
|---|---|---|---|---|---|---|
| Treat 3 | Treat 5 | Treat 7 | Treat 6 | Treat 2 | Treat 4 | Treat 1 |

Treatment groups:

Treat 1 represents Enteric-coated oral liposome/pCDNA3-LUC spray-dried microparticles vaccine.

Treat 2 represents Enteric-coated oral liposome/ pIHNV-G spray-dried microparticles vaccine.

Treat 3 represents Enteric-coated oral PEI/pCDNA3-LUC spray-dried microparticles vaccine.

Treat 4 represents Enteric-coated oral PEI/ pIHNV-G spray-dried microparticles vaccine.

Treat 5 represents Enteric-coated oral Eudragit® L-30 D spray-dried microparticles vaccine

Treat 6 represents pCDNA3-Luc intramuscular injection.

Treat 7 represents pIHNV-G intramuscular injection.

Distribution tanks: A1, A2, A3, A4, A5, A6

**Figure 4.6: Map of the tank for enteric-coated spray-dried microparticles experiment.**

Fish Vaccination Program

Fish were fed with regular feed or blank feed (vaccine free, Treatment 5) for 2 weeks prior to the treatment. In the oral treatment groups, fish in each tank were fed twice a day with 0.840 g of the enteric-coated spray-dried microparticle formulation (Treatment 1-4) and with 2 g of regular fish feed. Basically, each fish was expected to get 2 mg of microparticles feed containing 1 μg of plasmid DNA per day. Thus, the total amount of DNA received at day 14 of oral vaccination would be 14 μg DNA per fish. Fish in the oral control group were fed daily with approximately 3 g of blank feed. In the injected groups, fish (mean weight of 0.97 g) were anesthetized first by immersion with 50 mg/ml of 3-aminobenzoic acid ethyl ester (MS-222), Sigma Chemical Co. (St. Louis, MO, USA). Then each fish was vaccinated with 1 μg of pIHNV-G (Treatment 7) or pCDNA3-Luc (Treatment 6) by injecting 25 μl of the stock solution (40 μg of DNA/1 ml of phosphate buffer saline) into the base of the dorsal fin (Figure 4.7).

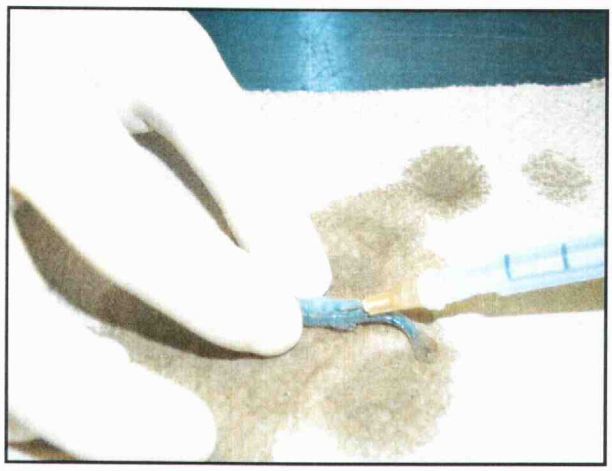


**Figure 4.7: Vaccination by injection route.** Rainbow trout fry was immunized by intramuscular injection at the base of dorsal fin.

*Immunization with Enteric-Coated Non-Pariel Sugar Beads DNA Vaccine*

Fish Distribution

Two hundred and sixty fish were randomly distributed into 2×100 liter tanks for the oral vaccine treatment groups, while one hundred and forty fish were randomly assigned into 2×100 liter tanks for the IM injection treatment groups. Tanks were randomly distributed in the region assigned by the number 8-11 (Figure 4.8). Fish were distributed into each tank at 25 fish at a time by randomly drawn cards until 25 fish were placed in all tanks. The cards were then reshuffled and another 25 fish were assigned again into each tank. The process was repeated until the numbers of fish in each tank reach 260 for oral treatment groups and 140 for IM injection groups.

| | | | |
|---|---|---|---|
| A8<br>Treat 9 | A9<br>Treat 11 | A10<br>Treat 10 | A11<br>Treat 8 |

Treatment groups:

Treat 8 represents Enteric-coated Oral PEI/pIHNV-G non-pariel sugar beads vaccine.

Treat 9 represents Enteric-coated Oral PEI non-pariel sugar beads vaccine.

Treat 10 represents pIHNV-G intramuscular injection.

Treat 11 represents PBS intramuscular injection.

Distribution tanks:

A1, A2, A3, A4, A5, A6

**Figure 4.8: Map of the tank for enteric-coated non-pariel sugar beads vaccine experiment.**

Fish Vaccination Program

Fish in IM injection groups and oral treatment groups were fed with regular feed or blank feed containing enteric-coated beads (Treatment 9), respectively for 2 weeks prior to the treatment. In the oral treatment groups, approximately 4 g of spray-coated feed (granules of enteric-coated PEI/pIHNV-G non-pariel sugar beads (Treatment 8) and regular fish feed) were given to fish in the tank. Each fish was expected to receive approximately 15.4 mg of spray-coated feed containing 0.35 μg of plasmid DNA per day. Fish in the oral control group were fed daily with approximately 4 g of blank feed. The total amount of DNA received at day 40 was 14 μg DNA per fish. In the injected groups, fish (mean weight of 0.97 g) were anesthetized first by immersion in 50 mg/ml of 3-aminobenzoic acid ethyl ester (MS-222), Sigma Chemical Co. (St. Louis, MO, USA). Fish were vaccinated with 1 μg of pIHNV-G (Treatment 10) by injecting 25 μl of the stock solution (40 μg DNA/ml of phosphate buffer saline) at the base of the dorsal fin (Figure 4.7). Fish in the IM injection control group were vaccinated with 25 μl of PBS (Treatment 11).

**Samples Collection and Preparation for Histology and Luciferase Analysis**

Samples of fish were collected at 1, 2, 4, and 6 week post vaccination for histology and luciferase analysis. At each time point, three fish were collected from the group treated with pIHNV-G and 6 fish were collected from the group treated with

pCDNA3-LUC and blank feed. Fish were sacrificed in 200 mg/ml of MS-222 before dissection. For histology analysis, the whole intestinal tract was dissected, put in the cassette, and placed in 10% buffered-formalin solution. Histology change in fish intestinal tract samples were analyzed by Dr. Jerry R. Heidel at College of Veterinary Medicine, Oregon State University (Corvallis, OR, USA). For luciferase analysis, the whole intestinal tract was dissected from fish and placed in a microcentrifuge tube followed immediately by freezing in dried ice. At the end of dissection, all intestinal samples were transferred from dried ice to -80 °C.

**Luciferase assay**

Luciferase expression in fish intestinal tract was examined by measuring the light production of luciferase using luciferase assay system protocol, Promega (Madison, WI, USA). The luciferase samples were prepared by grinding 1 volume of fish intestinal tract (100 µl ≈ 0.1 g tissue) with 2 volumes of phosphate buffer saline (PBS) containing protease inhibitor and 2 volume of 2X Passive lysis buffer (PLB), Promega (Madison, WI, USA), Table 4.13. The amount of protease inhibitor used is 5 µl per 0.1 g tissue. The resultant tissue samples were centrifuged at 15,000 rpm (12,576 ×g) for 1 minute. The supernatant was assayed for luciferase activity with luciferase assay reagent, Promega (Madison, WI, USA) according to the manufacture's instruction in a Lumat LB9507 luminometer, EG&G Berthold,

(Wilbach, Germany). Luciferase activity was expressed as relative light unit (RLU). To 20 µl of supernatant, 100 µl of luciferase assay reagent is added.

**Table 4.13: Luciferase sample preparation**

| Ingredients | Amount |
|---|---|
| Fish intestinal tissue | 1 volume (100 µl ≈ 0.1 g tissue) |
| Phosphate buffer saline (PBS) | 2 volume |
| Protease inhibitor | 5 µl |
| 2X Phosphate lysis buffer | 2 volume |

**Preliminary Virus Challenge Experiment**

Small fish trial was performed to determine the virus concentration needed in the lethal challenge. Fish were distributed randomly 10 at a time into three of 25 liter tanks, 50 fish per each tank. The water level of each fish tank was lowered to 1 liter. A virus concentration of $10^3$, $10^4$, and $10^5$ pfu IHNV per ml was added into one of the three tanks. Fish were exposed to the virus by bath immersion in the 1 liter volume along with air supply at 13 °C for 5 hours. After five hours exposure period, water flow was put back into the tank at the rate of 500 ml/hr and the mortalities were observed and a daily count of the mortalities kept for 30 days post challenge.

**Lethal Challenge by IHNV**

Fish Distribution

Fish from each oral treatment group were assigned into three duplicate 25 liter tanks with 50 fish per tank. No replication tanks were used for fish immunized by intramuscular injection. For the *in vivo* experiment of fish immunized by spray-coated feed, four duplicate tanks were employed for the oral treatment groups and two tanks were used for the IM injection treatment group. The additional tank was used as a negative control when exposing fish to the virus. A total of 17 tanks for enteric-coated spray-dried microparticle vaccine and 12 tanks for enteric-coated non-pariel sugar beads vaccine were used and distributed in a way that the tanks were placed within the region assigned by the letters A-F (Figure 4.9) and by the letters G-I (Figure 4.10). In each treatment, fish were distributed 10 at a time to the designated tank by randomly drawn cards until 10 fish were placed into each tank. The cards were then reshuffled and 10 fish were placed again into each of the three tanks. This process was repeated until the final amount of fish in each tank equaled fifty.

IHNV Challenge

After the immune development phase, fish were distributed into replicate tanks of 50 fish each as previous described. The *in vivo* challenge by IHNV was performed

at salmon disease laboratory. Briefly, Rainbow trout treated with enteric-coated spray-dried microparticles and enteric-coated non-pariel sugar beads were exposed respectively to $10^5$ pfu IHNV per ml (strain 220-90) and $10^4$ pfu IHNV/ml (strain IHNV-RA) diluted with phosphate buffer saline pH 7.4 by bath immersion with air supply for 5 hours (Figure 4.11). At the end of 5 hours, the water supply was allowed back into the tanks at the flow rate of 500 ml/hr. After virus challenge, fish were allowed feed daily, given trout pellets (strata # 1). Mortalities were observed daily for 30 days post IHNV challenge. Dead fish were counted, collected, and stored at -20 °C for further analysis. Plaque assay for IHNV was performed on approximately 10% of the dead fish. The rest of dead fish were stored at -20 °C and at the end of the study autoclaved and discarded.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| A1 Treat 5 | A2 Treat 5 | A3 Treat 5 | B1 Treat 1 | B2 Treat 1 | B3 Treat 1 | C1 Treat 4 | C2 Treat 4 | C3 Treat 4 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| D1 Treat 2 | D2 Treat 2 | D3 Treat 2 | E1 Treat 3 | E2 Treat 3 | E3 Treat 3 | F1 Treat 7 | F2 Treat 6 |

Treatment groups:

Distribution regions:

Treat 1 represents Enteric-coated oral liposome/pCDNA3-LUC spray-dried microparticles vaccine. A, B, C, D, E, F

Treat 2 represents Enteric-coated oral liposome/ pIHNV-G spray-dried microparticles vaccine.

Treat 3 represents Enteric-coated oral PEI/pCDNA3-LUC spray-dried microparticles vaccine.

Treat 4 represents Enteric-coated oral PEI/ pIHNV-G spray-dried microparticles vaccine.

Treat 5 represents Enteric-coated oral Eudragit® L-30 D spray-dried microparticles vaccine

Treat 6 represents pCDNA3-Luc intramuscular injection.

Treat 7 represents pIHNV-G intramuscular injection.

**Figure 4.9: Map of the tank for IHNV challenge of fish immunized with enteric-coated spray-dried microparticles vaccine.**

| G1 | G2 | G3 | G4 | H1 | H2 | H3 | H4 |
|---|---|---|---|---|---|---|---|
| Treat 11 | Treat 9 | Treat 8 | Treat 10 | Treat 8 | Treat 9 | Treat 8 | Treat 9 |

| I1 | I2 | I3 | I4 |
|---|---|---|---|
| Treat 8 | Treat 11 | Treat 10 | Treat 9 |

Treatment groups:

Treat 8 represents Enteric-coated Oral PEI/pIHNV-G non-pariel sugar beads vaccine.

Treat 9 represents Enteric-coated Oral PEI non-pariel sugar beads vaccine.

Treat 10 represents pIHNV-G intramuscular injection.

Treat 11 represents PBS intramuscular injection.

Distribution tanks:

G, H, I

**Figure 4.10: Map of the tank for IHNV challenge of fish immunized with enteric-coated non-pariel sugar beads vaccine**

**Figure 4.11: Vaccination of Rainbow trout fry with IHNV by bath immersion.** Fish were challenged in a group of 50 fish with IHNV. Fish were exposed to the virus by bath immersion in the 1 liter volume along with air supply at 13 °C for 5 hours.

**Tissue IHNV Isolation**

First, whole Rainbow trout was placed into plastic stomacher bag, Fisher Scientific Company (Pittsburgh, PA, USA) followed by adding with 1X HBSS (Hanks' Balanced Salt Solution), Life Technologies Inc. (Rockville, MD, USA) containing 10% antibiotic antimycotic solution. The volume of 1X HBSS needed was determined by multiplying the weights of fish by 9. Second, the fish sample was

mashed for about 20 second or until the mass was sufficiently broken up using stomacher 80 from Colworth. Lastly, the liquid mass was poured into a 15 ml tube and refrigerated overnight. After 24 hours, virus samples were collected by centrifugation at 4 °C, 3,000 rpm (956 ×g) for 15 minutes and plaque assay was performed.

**Plaque Assay for IHNV**

The supernatant from the liquid mass of whole Rainbow trout was diluted in 10-fold stepwise process, $10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$, with MEM. One hundred microliters of each dilution and the non-diluted sample was added into the confluent monolayer of CHSE-214 cells grown in 12 well plates. Cells were incubated with the virus sample at room temperature for 2 hours. Two milliliters of methylcellulose overlay media overlay was added into each well after 1 hour. Then, cells were incubated for at least 7 days in a $CO_2$ chamber at 17 °C followed by staining with 1 milliliter of crystal violet-formalin solution (see appendix) overnight. The stained plates were rinsed with tap water, air-dried, and the number of plaques were counted. The viral concentration was determined as followed:

Viral concentration (pfu/ml) = Number of plaque ×dilution factor ×1/volume in ml.

equation (1)

## RESULTS AND DISCUSSION

### Preparation of Liposome-DNA and PEI-DNA Complexes Embedding in Microparticles using Spray Drying Technique

Previous results from our laboratory suggested that liposome and polyethylenimine (PEI) work as a plasmid DNA carrier to help deliver plasmid DNA into cells resulting in gene expression in cells. Additionally, degradation of plasmid DNA by the harsh environment in the GUT can be prevented by embedding the plasmid DNA into enteric protected microparticles composed of Eudragit® L-30 D. To obtain successful oral DNA delivery into cells, liposome/DNA or PEI/DNA complexes embedded in enteric protected microparticles were produced by combining these two techniques together. The structural integrity of plasmid DNA (pCDNA3-Luc) as determined from agarose gel electrophoresis showed that the extracted plasmid DNA remained intact in the microparticles; both relaxed and supercoiled forms were observed (Figure 4.12). Functional integrity of extracted plasmid DNA from transfection assay in CHSE-214 cells revealed that extracted plasmid DNA from the microparticles was able to transfect cells; however, the transfection efficiency tends to be less than the control plasmid DNA with the same transfecting reagent, Lipofectin®. The percent luciferase count per second (LCPS) is 62.63 % for extracted plasmid DNA and 100 % for control plasmid DNA (Figure 4.13). This might due to the fact that during the microparticles preparation process, plasmid DNA looses some

of its structural integrity as a higher proportion of relaxed form was observed in agarose gel compared to the control DNA. The supercoiled was believed to have a higher efficiency in achieving cell transfection.

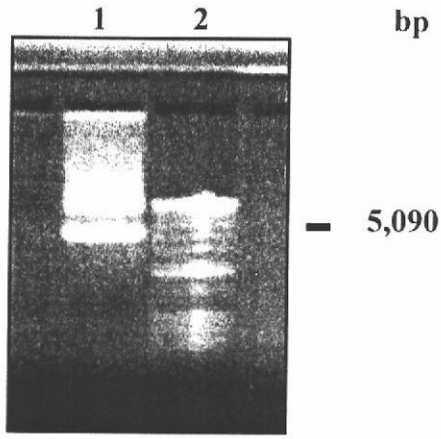



**Figure 4.12: Agarose gel electrophoresis of extracted DNA from Eudragit® L-30 D spray-dried microparticles embedded with liposome/pCDNA3-LUC.** Lane 1 is extracted pCDNA3-LUC in the amount of ~0.2 μg. Lane 2 is DNA ladder.

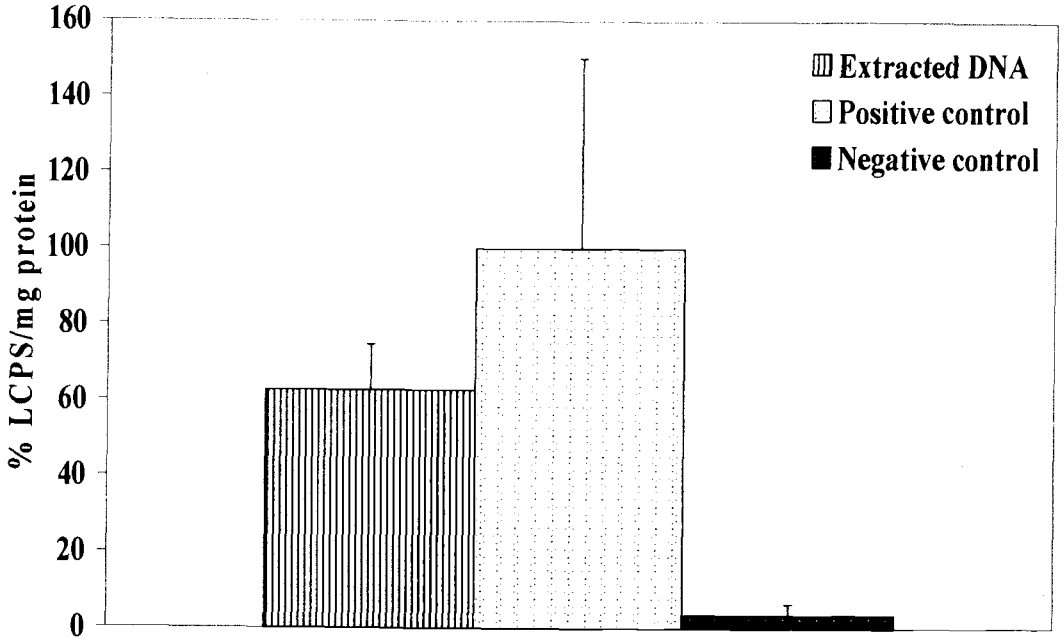



**Figure 4.13: Gene expression of extracted pCDNA3-LUC in CHSE-214 cells.** CHSE-214 cells were transfected with lipofectin® complexed with control pCDNA3-LUC (positive control) and with extracted pDNA3-LUC (~1 μg per well). Cell treated with naked DNA served as negative control. The luciferase activity was reported as percent luciferase count per second (LCPS) per mg protein. Mean ± SD (n=3).

**Table 4.14: Gene expression of extracted pCDNA3-LUC in CHSE-214 cells**

| Treatment | %LCPS/mg protein | SD |
|---|---|---|
| Extracted DNA | 62.63 | 12.02 |
| Positive control | 100 | 50.20 |
| Negative control | 3.61 | 2.83 |

**Immunization with Enteric-Coated Spray-Dried Microparticles DNA Vaccine**

Fish were trained to eat enteric-coated spray-dried Eudragit® L-30 D microparticles (Blank feed, Treatment 5) for 3 weeks prior to the vaccine treatment feed (Treatments 1-4). Mortalities and the average weight of dead fish were observed during the treatment feeding. The total number of dead fish at the end of week four was 3, 3, 4, 22, 12, 13, and 17 for treatment groups 1 through 7, respectively. The average weight of the dead fish was 0.877, 0.739, 0.9, 0.83, 0.91, and 0.91 g for treatment groups 1 through 7 (Table 4.15). These mortalities might be due to the lack of nutrients in the feed since treatment feed contained a mixture of microparticles and autoclaved regular feed which might have lost some of its nutritious value under the high temperature of the autoclaving process. Additionally, a lower amount of feed was consumed by the fish when the feed was changed from the regular feed to the blank feed. Approximately 2 g of regular fish feed was given additionally to fish in each tank to substitute for the nutrition loss.

Fish were given the oral vaccine treatment (Treatments 1-4) or the intramuscular injection vaccine treatment (Treatments 6-7) at the end of third week. Fish in all treatment groups ate energetically. Fish immunized by intramuscular administration showed some swelling and discoloration at the injection site within the first 24 hours after injection. No sign of swelling and discoloration was observed after 48 hours.

**Table 4.15: Mortalities and average weight of dead fish during viral challenge trial after vaccination with enteric-coated spray-dried microparticles vaccine**

| Treatment[a] | Mortalities | Average weight (g) |
|---|---|---|
| 1 | 3 | 0.877 |
| 2 | 3 | 0.852 |
| 3 | 4 | 0.739 |
| 4 | 22 | 0.9 |
| 5 | 12 | 0.83 |
| 6 | 13 | 0.91 |
| 7 | 17 | 0.91 |

[a]Treatment 1 represents Enteric-coated oral liposome/pCDNA3-LUC spray-dried microparticles vaccine.
Treatment 2 represents Enteric-coated oral liposome/ pIHNV-G spray-dried microparticles vaccine.
Treatment 3 represents Enteric-coated oral PEI/pCDNA3-LUC spray-dried microparticles vaccine.
Treatment 4 represents Enteric-coated oral PEI/ pIHNV-G spray-dried microparticles vaccine.
Treatment 5 represents Enteric-coated oral Eudragit® L-30 D spray-dried microparticles vaccine
Treatment 6 represents pCDNA3-Luc intramuscular injection.
Treatment 7 represents pIHNV-G intramuscular injection.

Luciferase and Histology Analysis

The luciferase activity from the homogenized digestive tract samples could not be detected by luciferase assay system, Promega (Madison, WI, USA). In fish given enteric-coated oral liposome/pCDNA3-LUC spray-dried microparticles vaccine (Treatment 1) and enteric-coated oral PEI/pCDNA3-LUC spray-dried microparticles vaccine (Treatment 3), the percent relative light unit (RLU) showed a value of 0.002% and 0.0044% of the percent RLU from the muscle tissue of the positive control (Treatment 6) equal 100%. Also, some of the samples contained high background and could not be detected by the luminometer due to the limitation of the machine.

No histological change in fish intestinal tract was observed during the histology analysis. There was no abnormality seen in the intestinal tissue samples except an occasional incidental change in a few of the specimens (a few old granulomas and foci of mineralization, probably from old parasite migration). However, this result also revealed that the enteric protection polymer of the microparticles (Eudragit® L-30 D) is not harmful to fish cells.

Lethal Challenge with IHNV

It is important to determine the concentration of virus used in the lethal challenge experiment. Low virus concentration will create virus resistance to the fish and too high of a virus concentration tends to kill most of the fish including those that

have already created an immune response against the disease. The optimum virus concentration needed should be able to kill 70-80% of the total fish.

From the preliminary virus challenge experiment, the percent mortality after virus challenge was 14%, 30%, and 76% for fish treated with $10^3$, $10^4$, and $10^5$ pfu IHNV per ml virus concentration (Figure 4.14). Thus, the optimum virus concentration of $10^5$ pfu IHNV per ml was chosen in the lethal virus challenge experiment.

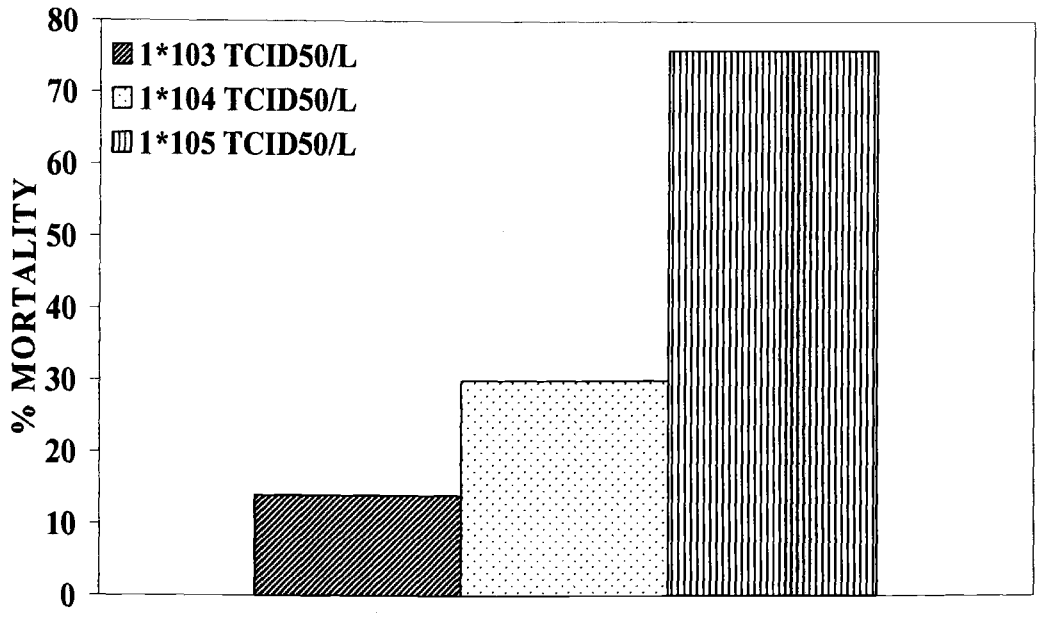



**Figure 4.14: Virus concentration for IHNV challenge of fish immunized with enteric-coated spray-dried microparticles vaccine.** Graph shows cumulative percent mortality for the 30 day challenge. Fish were challenged in a group of 50 by immersion in $10^3$, $10^4$, and $10^5$ pfu/ml IHNV strain 220-90. Mortalities were collected daily for 30 days. The optimum virus concentration for lethal challenge experiment was $10^5$ pfu IHNV per ml.

**Table 4.16: Virus concentration for IHNV challenge of fish immunized with enteric-coated spray-dried microparticles vaccine**

| Virus concentration (pfu/ml) | Percent mortality |
|---|---|
| $10^3$ | 14 |
| $10^4$ | 30 |
| $10^5$ | 76 |

The mortalities observed 30 days after virus challenge showed that there was no significant difference between groups treated with treatment 1 through treatment 6 (p-value < 0.3175, chi square test) (see Table 4.18 for treatment groups). The percent mortalities are 82.67%, 84%, 82.67%, 92%, 86%, and 80% for treatment 1 through treatment 6, respectively (Figure 4.15 and Table 4.18). The percent mortality of fish treated with IM injection of pIHNV-G plasmids (treatment 7) was 4% which showed significant protection against IHNV ($p < 0.0001$, chi square test). Statistical analysis also showed that there was no significant difference in the number of mortalities between the individual tanks ($P > 0.05$, chi square test, Table 4.18), thus tank observations were disregarded and treatment groups of 150 fish per group were evaluated. The symptoms of IHNV, darkened body coloration, vertical drifting, distended abdomens, and pop eye were also observed in the dead fish (Figure 4.16). Plaque assay performed on 10% of the dead fish verified that the mortalities of the fish were caused by IHNV (Figure 4.17). Less than 0.7% of the total number of mortalities was due to extraneous causes.

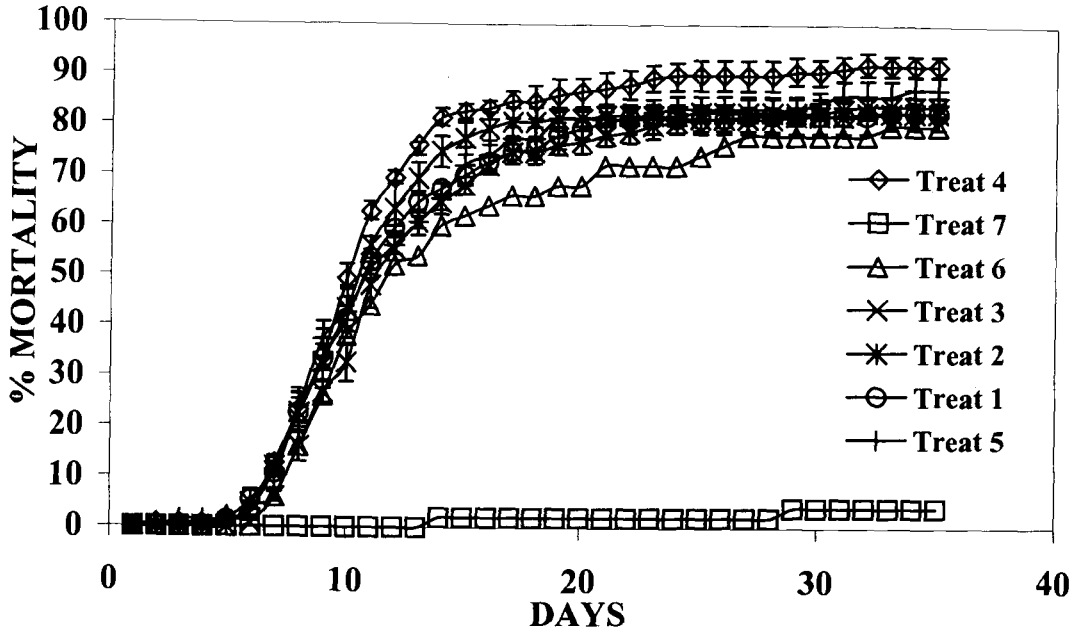



**Figure 4.15: IHNV challenge results from enteric-coated spray-dried microparticle vaccination.** Cumulative percent mortality curves for fish challenged 6 weeks after oral vaccination with enteric-coated spray-dried microparticles vaccine. Fish were challenged in a group of 50 fish with $10^5$ pfu/ml IHNV strain 220-90. Mortalities were observed for 30 days post challenge. Triplicate tanks of 50 fish were used for oral treatment group. Single tank of 50 fish was used for IM injection treatment group.

Treat 1: Enteric-coated oral liposome/pCDNA3-LUC spray-dried microparticles vaccine.
Treat 2: Enteric-coated oral liposome/ pIHNV-G spray-dried microparticles vaccine.
Treat 3: Enteric-coated oral PEI/pCDNA3-LUC spray-dried microparticles vaccine.
Treat 4: Enteric-coated oral PEI/ pIHNV-G spray-dried microparticles vaccine.
Treat 5: Enteric-coated oral Eudragit® L-30 D spray-dried microparticles vaccine.
Treat 6: pCDNA3-Luc intramuscular injection.
Treat 7: pIHNV-G intramuscular injection.

**Table 4.17: IHNV challenge results from enteric-coated spray-dried microparticle vaccination**

| | Daily percent mortality rate | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Day | Treat 1 | SD | Treat 2 | SD | Treat 3 | SD | Treat 4 | SD | Treat 5 | SD | Treat 6 | Treat 7 |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0.67 | 0.58 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0.67 | 0.58 | 1.33 | 0.58 | 0 | 0 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0.67 | 0.58 | 1.33 | 0.58 | 0 | 0 |
| 5 | 1.33 | 1.15 | 0 | 0 | 0 | 0 | 1.33 | 1.15 | 1.33 | 0.58 | 2 | 0 |
| 6 | 5.33 | 2.08 | 4.67 | 2.52 | 0.67 | 0.58 | 2.67 | 1.53 | 3.33 | 1.53 | 4 | 0 |
| 7 | 10.67 | 3.06 | 12.00 | 1.73 | 6.00 | 1.00 | 12.67 | 2.08 | 11.33 | 1.53 | 6 | 0 |
| 8 | 22.67 | 3.79 | 22.67 | 4.04 | 16.00 | 3.00 | 23.33 | 3.51 | 24.00 | 3.46 | 16 | 0 |
| 9 | 33.33 | 5.86 | 32.67 | 3.51 | 26.67 | 2.08 | 34.00 | 3.00 | 37.33 | 3.51 | 26 | 0 |
| 10 | 41.33 | 2.52 | 43.33 | 2.31 | 32.67 | 3.79 | 49.33 | 2.08 | 42.67 | 5.13 | 38 | 0 |
| 11 | 52.67 | 2.52 | 56.00 | 1.73 | 48.00 | 4.58 | 62.67 | 1.53 | 51.33 | 2.08 | 44 | 0 |
| 12 | 59.33 | 0.58 | 63.33 | 4.93 | 55.33 | 2.31 | 69.33 | 0.58 | 56.00 | 1.73 | 52 | 0 |
| 13 | 64.67 | 2.08 | 69.33 | 3.21 | 60.67 | 1.15 | 76.00 | 2.00 | 60.67 | 2.52 | 54 | 0 |
| 14 | 67.33 | 1.15 | 74.67 | 3.06 | 64.67 | 1.53 | 81.33 | 1.53 | 65.33 | 3.06 | 60 | 2 |
| 15 | 70.00 | 2.00 | 77.33 | 3.21 | 68.00 | 1.73 | 82.67 | 1.53 | 72.00 | 3.61 | 62 | 2 |
| 16 | 72.67 | 1.53 | 79.33 | 3.21 | 72.00 | 1.73 | 83.33 | 2.08 | 74.00 | 3.00 | 64 | 2 |
| 17 | 74.67 | 1.53 | 80.67 | 2.89 | 74.67 | 2.31 | 84.67 | 3.21 | 77.33 | 2.31 | 66 | 2 |
| 18 | 75.33 | 2.08 | 80.67 | 2.89 | 74.67 | 2.31 | 84.67 | 3.21 | 77.33 | 2.31 | 66 | 2 |
| 19 | 78.00 | 2.65 | 81.33 | 2.31 | 76.00 | 1.73 | 86.00 | 2.65 | 81.33 | 1.53 | 68 | 2 |
| 20 | 79.33 | 2.08 | 81.33 | 2.31 | 76.67 | 2.31 | 86.67 | 3.21 | 81.33 | 1.53 | 68 | 2 |
| 21 | 80.67 | 3.21 | 82.00 | 1.73 | 78.00 | 2.00 | 87.33 | 3.06 | 81.33 | 1.53 | 72 | 2 |
| 22 | 81.33 | 2.89 | 82.00 | 1.73 | 78.67 | 2.08 | 88.00 | 2.65 | 81.33 | 1.53 | 72 | 2 |
| 23 | 81.33 | 2.89 | 82.00 | 1.73 | 80.00 | 2.65 | 89.33 | 2.52 | 82.67 | 2.52 | 72 | 2 |

**Table 4.17: IHNV challenge results from enteric-coated spray-dried microparticle vaccination (continued)**

| | Daily percent mortality rate | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Day** | **Treat 1** | **SD** | **Treat 2** | **SD** | **Treat 3** | **SD** | **Treat 4** | **SD** | **Treat 5** | **SD** | **Treat 6** | **Treat 7** |
| 24 | 81.33 | 2.89 | 82.00 | 1.73 | 80.67 | 2.08 | 90.00 | 3.00 | 83.33 | 2.52 | 72 | 2 |
| 25 | 81.33 | 2.89 | 82.67 | 1.15 | 81.33 | 2.31 | 90.00 | 3.00 | 83.33 | 2.52 | 74 | 2 |
| 26 | 81.33 | 2.89 | 82.67 | 1.15 | 81.33 | 2.31 | 90.00 | 3.00 | 83.33 | 2.52 | 76 | 2 |
| 27 | 82.00 | 3.46 | 82.67 | 1.15 | 81.33 | 2.31 | 90.00 | 3.00 | 83.33 | 2.52 | 78 | 2 |
| 28 | 82.00 | 3.46 | 82.67 | 1.15 | 81.33 | 2.31 | 90.00 | 3.00 | 83.33 | 2.52 | 78 | 2 |
| 29 | 82.00 | 3.46 | 82.67 | 1.15 | 82.00 | 1.73 | 90.67 | 2.52 | 83.33 | 2.52 | 78 | 4 |
| 30 | 82.00 | 3.46 | 82.67 | 1.15 | 82.00 | 1.73 | 90.67 | 2.52 | 84.67 | 2.08 | 78 | 4 |
| 31 | 82.00 | 3.46 | 83.33 | 1.53 | 82.00 | 1.73 | 91.33 | 2.52 | 86.00 | 3.00 | 78 | 4 |
| 32 | 82.00 | 3.46 | 84.00 | 1.73 | 82.67 | 1.15 | 92.00 | 2.00 | 86.00 | 3.00 | 78 | 4 |
| 33 | 82.67 | 3.21 | 84.00 | 1.73 | 82.67 | 1.15 | 92.00 | 2.00 | 86.00 | 3.00 | 80 | 4 |
| 34 | 82.67 | 3.21 | 84.00 | 1.73 | 82.67 | 1.15 | 92.00 | 2.00 | 87.33 | 2.52 | 80 | 4 |
| 35 | 82.67 | 3.21 | 84.00 | 1.73 | 82.67 | 1.15 | 92.00 | 2.00 | 87.33 | 2.52 | 80 | 4 |

Treat 1: Enteric-coated oral liposome/pCDNA3-LUC spray-dried microparticles vaccine.
Treat 2: Enteric-coated oral liposome/ pIHNV-G spray-dried microparticles vaccine.
Treat 3: Enteric-coated oral PEI/pCDNA3-LUC spray-dried microparticles vaccine.
Treat 4: Enteric-coated oral PEI/ pIHNV-G spray-dried microparticles vaccine.
Treat 5: Enteric-coated oral Eudragit® L-30 D spray-dried microparticles vaccine.
Treat 6: pCDNA3-Luc intramuscular injection.
Treat 7: pIHNV-G intramuscular injection.

**Table 4.18: Mortality of fish after IHNV challenge**

| Treatment | Total number of fish per group | Number of dead fish | | | Total Number of dead fish | Average percent mortality | p-value |
|---|---|---|---|---|---|---|---|
| | | Tank1 | Tank2 | Tank3 | | | |
| 1 | 150 | 45 | 39 | 40 | 124 | 82.67 | 0.236 |
| 2 | 150 | 43 | 43 | 40 | 126 | 84 | 0.640 |
| 3 | 150 | 42 | 40 | 42 | 124 | 82.67 | 0.830 |
| 4 | 150 | 48 | 44 | 46 | 138 | 92 | 0.337 |
| 5 | 150 | 41 | 44 | 46 | 131 | 87.33 | 0.318 |
| 6 | 50 | 40 | - | - | 40 | 80 | - |
| 7 | 50 | 2 | - | - | 2 | 4 | - |

Treatment groups:

Treat 1 represents Enteric-coated oral liposome/pCDNA3-LUC spray-dried microparticles vaccine.

Treat 2 represents Enteric-coated oral liposome/ pIHNV-G spray-dried microparticles vaccine.

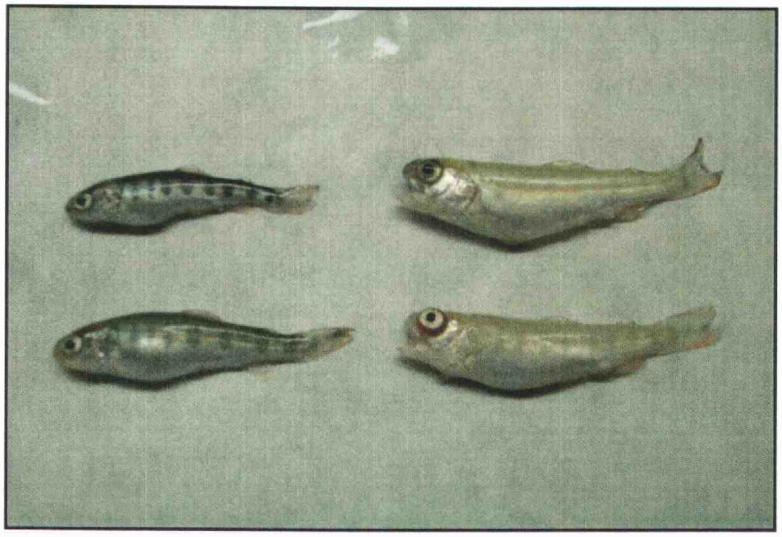
Treat 3 represents Enteric-coated oral PEI/pCDNA3-LUC spray-dried microparticles vaccine.

Treat 4 represents Enteric-coated oral PEI/ pIHNV-G spray-dried microparticles vaccine.

Treat 5 represents Enteric-coated oral Eudragit® L-30 D spray-dried microparticles vaccine

Treat 6 represents pCDNA3-Luc intramuscular injection.

Treat 7 represents pIHNV-G intramuscular injection



(a)

(b)

**Figure 4.16: Classic symptoms caused by IHNV.** Panel A shows a discoloration of fish body. Panel B shows a pop eye.

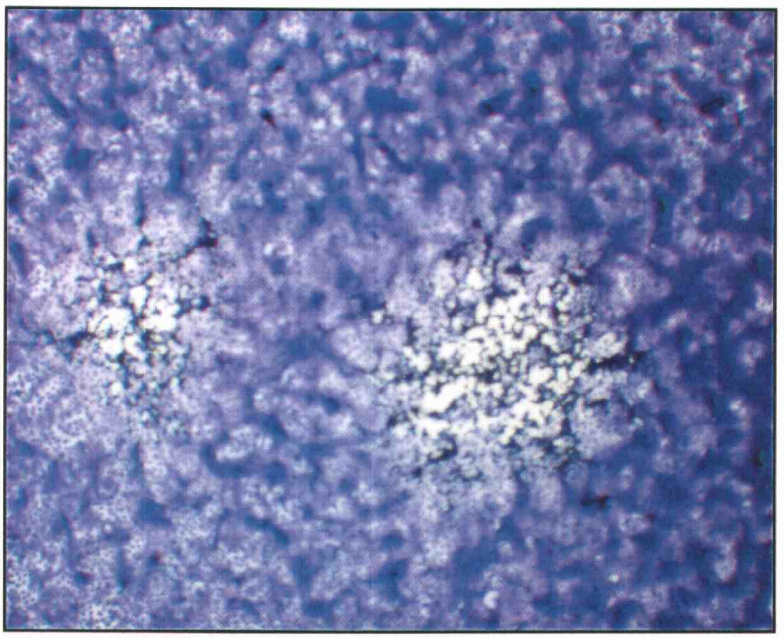


**Figure 4.17: Plaque caused by IHNV.**

Initial results from *in vivo* experiments showed that the lack of efficacy in the oral vaccine of spray-dried microparticles is more likely due to the premature release of plasmid DNA before it reaches the site of action and is subsequently destroyed by GI enzymes and the acidic environment in the GUT. While Eudragit® L-30 D is a pH dependent polymer that dissolves at the pH > 5.5, during the feeding process, microparticles in the feed dissolve when the enteric-protection polymer comes in contact with the water (pH 7.4) if the particle size of microparticles is too small and then enteric-protected polymer cannot protect the vaccine as it will erode away leaving the DNA. To reduce the amount of polymer from eroding or dissolving off the

microparticles before being consumed by fish, the fish were trained to eat blank feed mixed with polymer only microparticles for 3 weeks before given the treatment feed. The observation of a white mass of the polymer microparticles from the feed in the fish stomach confirmed that microparticles containing plasmid DNA was being eaten. One of the most important factors that will allow microparticles to reach the site of action in the intestine is the size of pyloric sphincter. Immunization will occur when the feed passes through the pyloric sphincter of the stomach and reaches the site of action in the intestine. The average size of pyloric sphincter in the fish used in the experiment was about 0.5-0.8 mm which is much larger than the size of microparticles used in the study. However, upon contact with the water in the stomach, microparticles tend to swell and stick together due to their close proximity to each other in the feed resulting in increasing the size of the microparticles in the feed. The swollen microparticles in the feed (> 0.8 mm) were not able to pass through the pyloric sphincter and eventually, the polymer undergoes self-erosion resulting in release of the plasmid DNA in to the stomach. Once released, the plasmid DNA will be destroyed by the harsh environment in the stomach.

Spray-dried enteric protected non-pariel sugar beads (40-50 mesh) containing plasmid DNA were investigated. Being larger in size and fewer of them, if the polymer coating swells the particles should not be able to stick together due to the distance separating them. Granulation of regular feed with the larger size of particles should prevent the particles from sticking to one another when they contact water, thus

passing through the pyloric sphincter and increasing the opportunity for the DNA to reach the site of action to induce an immune response. The *in vivo* experiments using spray-coated enteric protected beads were performed to determine the protective ability of this delivery system against IHNV.

**Preparation of Enteric-Coated Non-Pariel Sugar Beads Containing PEI/DNA Complexes using Spray Coating Technique**

Enteric-coated beads containing PEI/DNA complexes were prepared using the spray coating technique. The *in vitro* dissolution profile showed no DNA released from spray-coated bead within the first 2 hours in simulated gastric fluid (pH 1.23) followed by an immediate release in simulated intestinal fluid (pH 7.4) (Figure 4.18).

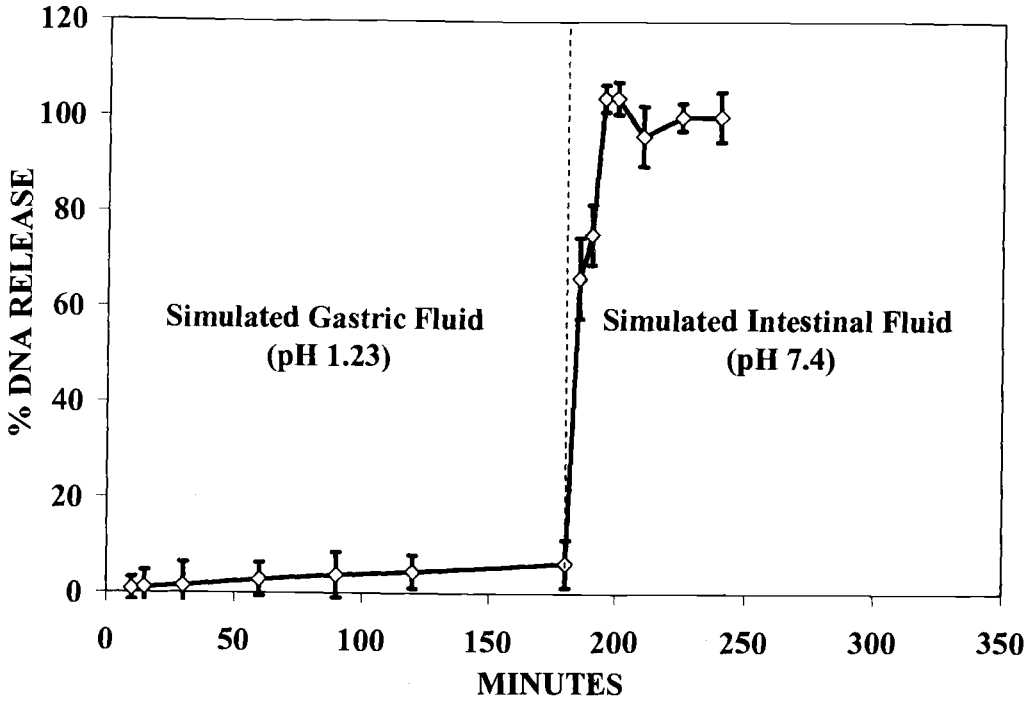



**Figure 4.18: The *in vitro* dissolution profile of pIHNV-G from enteric-coated beads.** Percentage of entrapped pIHNV-G release from 0.4 g enteric-coated beads (135.7 µg of pIHNV-G) was examined by dissolution test for 3 hours in simulated gastric fluid (SGF) pH 1.23 followed by 1 hour in simulated intestinal fluid (SIF) pH 7.4. Mean ± SD (n=3).

**Table 4.19: The *in vitro* dissolution profile of pIHNV-G from enteric-coated beads**

| Time (minutes) | % pIHNV-G release[a] | SD |
|---|---|---|
| 10 | 0.87 | 2.39 |
| 15 | 1.09 | 3.55 |
| 30 | 1.49 | 4.85 |
| 60 | 2.78 | 3.50 |
| 90 | 3.76 | 4.75 |
| 120 | 4.44 | 3.49 |
| 180 | 6.26 | 5.02 |
| 185 | 66.06 | 8.49 |
| 190 | 75.28 | 6.32 |
| 195 | 103.85 | 2.82 |
| 200 | 103.91 | 3.35 |
| 210 | 95.95 | 6.32 |
| 225 | 100.00 | 2.89 |
| 240 | 99.96 | 5.21 |

[a]Percentage of entrapped pIHNV-G release from 0.4 g enteric-coated beads (135.7 μg of pIHNV-G) was examined by dissolution test for 3 hours in simulated gastric fluid (SGF) pH 1.23 followed by 1 hour in simulated intestinal fluid (SIF) pH 7.4. Mean ± SD (n=3).

## Immunization by Enteric-Coated Non-Pariel Sugar Beads Vaccine

Fish in the oral treatment group were trained with enteric-coated PEI non-pariel sugar beads (Blank feed, Treatment 9) for 2 weeks prior to vaccination. At the end of second week, fish were vaccinated with either oral treatment (Treatments 8 and 9) or intramuscular injection treatment (Treatments 10 and 11) of vaccine. The mortalities and the average weight of dead fish were recorded during the treatment feed. The total amount of dead fish at the end of week six is 9, 5, 15, and 7 for

treatments 8 through 11, respectively (Table 4.20). The average weight of the dead fish is 0.52, 0.47, 0.63, and 0.57 (Table 4.20).

**Table 4.20: Mortalities and average weight of dead fish during vaccination with enteric-coated non-pariel sugar beads vaccine**

| Treatment[a] | Mortalities | Average weight (g) |
|---|---|---|
| 8 | 9 | 0.52 |
| 9 | 5 | 0.47 |
| 10 | 15 | 0.63 |
| 11 | 7 | 0.57 |

[a]Treat 8 represents Enteric-coated Oral PEI/pIHNV-G non-pariel sugar beads vaccine.
Treat 9 represents Enteric-coated Oral PEI non-pariel sugar beads vaccine.
Treat 10 represents pIHNV-G intramuscular injection.
Treat 11 represents PBS intramuscular injection.

Mortalities observed during vaccination strongly depend on the lack of nutrition for the oral group and the amount of traumatic injury to the fish for the injection group. Previous *in vivo* experiments suggest that lack of nutrition in the feed due to autoclaving feed was one of the reasons causing death. Thus, in this *in vivo* trial, regular fish feed was used instead of the autoclaved feed. However, during fish distribution process (placing fish into the different tank), fish were subjected to a stressful environment causing a lost of energy and appetite in some fish. It was noted that after the distribution process of fish into the tanks, a few fish stayed at the bottom

of the tank and did not consume any feed at all. This effect produced the lack of nutrition seen in those fish and consequently the death of the fish.

Luciferase Analysis

Similar to the results obtained from fish vaccinated with enteric-coated spray-dried microparticles vaccine, the luciferase activity from homogenized digestive tract samples could not be detected with the luciferase assay system using a luminometer.

Lethal Challenge with IHNV

Preliminary virus challenge experiment demonstrated that the optimum virus concentration was found to be $10^4$ pfu IHNV per ml. Percent mortality after virus challenge was found to be 38%, 75%, 90% for fish exposed to $10^3$, $10^4$, $10^5$ pfu IHNV per ml virus concentration (Figure 4.19). A lower virus concentration was used in the challenge experiment of fish vaccinated with enteric-coated non-pariel sugar beads DNA vaccine ($10^4$ pfu/ml IHNV) compared to fish vaccinated with enteric-coated spray-dried microparticles DNA vaccine ($10^5$ pfu/ml IHNV). This was possibly due to a difference in virus strains used as well as the susceptibility of fish to the virus.

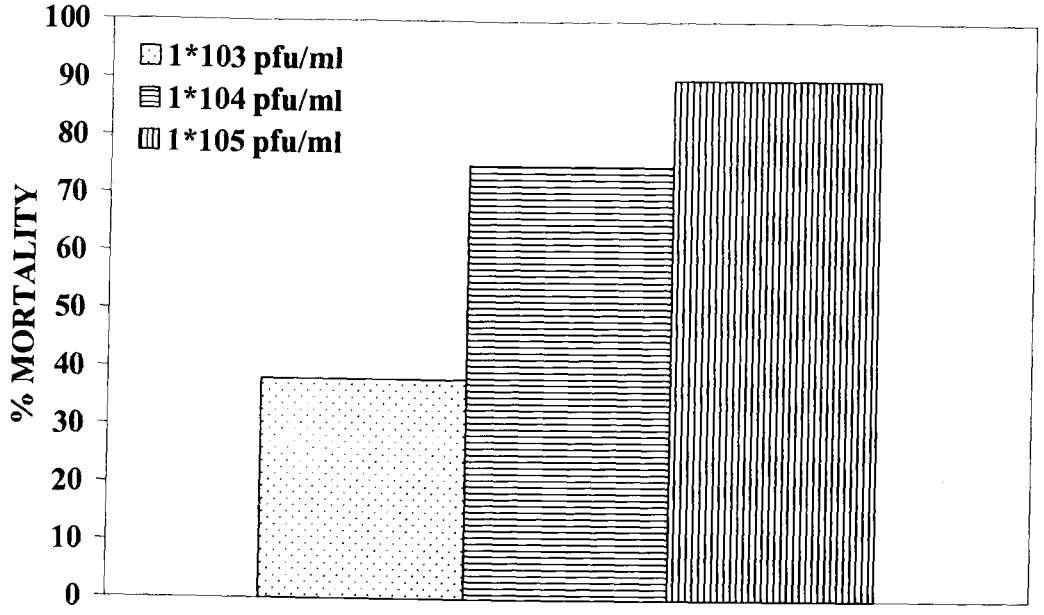



**Figure 4.19: Virus concentration for IHNV challenge of fish immunized with enteric-coated non-pariel sugar beads DNA vaccine.** Graph shows cumulative percent mortality for the 30 day challenge. Fish were challenged in a group of 50 by immersion in $10^3$, $10^4$, and $10^5$ pfu/ml IHNV strain IHNV-RA. Mortalities were collected daily for 30 days. The optimum virus concentration for lethal challenge experiment was $10^4$ pfu IHNV per ml.

**Table 4.21: Virus concentration for IHNV challenge of fish immunized with enteric-coated spray-dried microparticles vaccine**

| Virus concentration (pfu/ml) | Percent mortality |
|---|---|
| $10^3$ | 38 |
| $10^4$ | 75 |
| $10^5$ | 90 |

The mortalities observed 30 days after virus challenge showed a significant difference between oral treatment group (Treatment 8) and the oral blank group

(Treatment 9), (p-value < 0.002, chi square test). The percent mortalities were 55.33% and 72.67% for treatment 8 and treatment 9, respectively (Figure 4.20 and Table 4.23). This result suggests an improvement in the protection against IHNV when fish were immunized with spray-coated beads. The fish mortality observed from the oral immunization group, however, was not comparable to the IM injection group. A higher level of protection against IHNV was observed in fish vaccinated by intramuscular injection route compared to fish vaccinated by oral administration (p-value < 0.0001, chi square test). The percent mortalities were 6% and 55.33% for injection group (Treatment 10) and oral treatment group (Treatment 8), respectively (Figure 4.20 and Table 4.23).

Fish vaccinated with PBS solution (Treatment 11) had less mortalities compared to fish vaccinated with oral treatment vaccine feed (Treatment 8). Statistical analysis, however, suggested no significant difference between these two treatment groups (p-value > 0.512, chi square test). The percent mortalities were 50% and 55.33% for treatment 11 and treatment 8, respectively (Figure 4.20 and Table 4.23).

The results of the similar mortalities of oral blank group (treatment 9) and PBS injection group (Treatment 11) was unexpected. The low mortality rate of the PBS injection group was likely due to the larger size of fish in the PBS injection group. Larger fish are more resistant to IHNV viral infection compared to small fish. The average size of fish in the oral group and the PBS injection group was approximately

0.86 and 1.02 g, respectively. Additionally, fish in the PBS injection group were kept in one tank and the mortalities observed in the injection group came from this one tank while three replicate tanks was used for the oral vaccine and blank treatment groups. Any conclusion to compare the mortalities between the two control groups can not be made from these results. Further experiment using three replicate tanks in all groups are suggested.

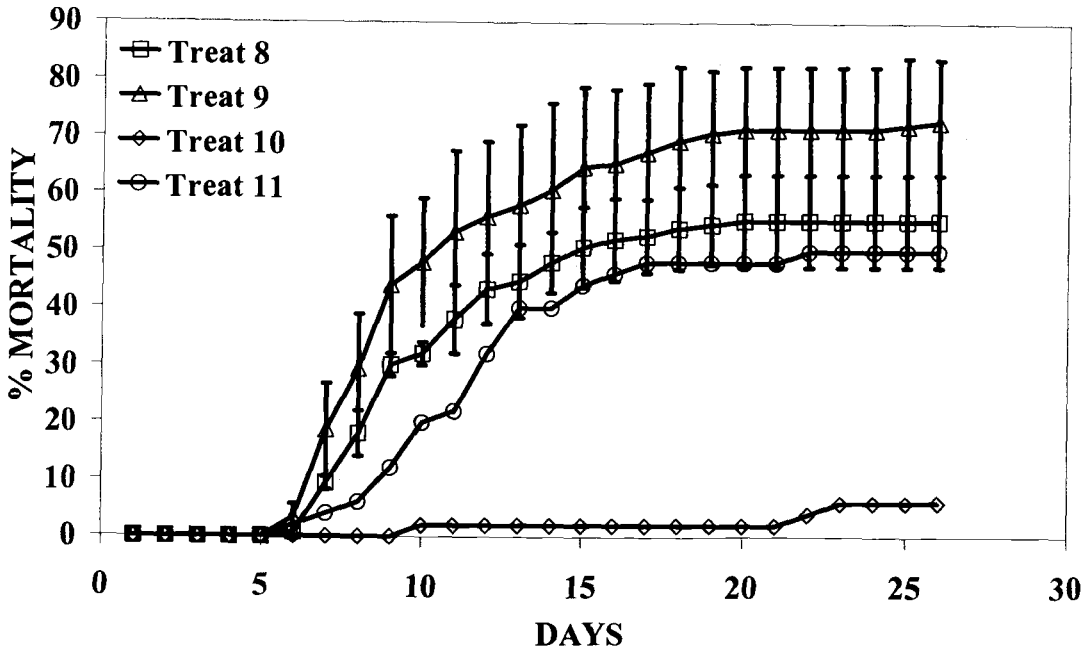



**Figure 4.20: IHNV challenge results from enteric-coated non-pariel beads vaccination.** Cumulative percent mortality curves for fish challenged 6 weeks after oral vaccination with enteric-coated spray-dried microparticles vaccine. Fish were challenged in a group of 50 fish with $10^4$ pfu IHNV per ml. Mortalities were observed for 30 days post challenge. Triplicate tanks of 50 fish were used for oral treatment group. Single tank of 50 fish was used for IM injection treatment groups.

Treat 8 represents Enteric-coated Oral PEI/pIHNV-G non-pariel sugar beads vaccine.
Treat 9 represents Enteric-coated Oral PEI non-pariel sugar beads vaccine.
Treat 10 represents pIHNV-G intramuscular injection.
Treat 11 represents PBS intramuscular injection.

**Table 4.22: IHNV challenge results from enteric-coated non-pariel beads vaccination**

| Day | Daily percent mortality rate | | | | | |
|---|---|---|---|---|---|---|
| | Treat 8 | SD | Treat 9 | SD | Treat 10 | Treat 11 |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 0.67 | 1.15 | 3.33 | 2.31 | 0 | 2 |
| 7 | 9.33 | 1.15 | 18.67 | 8.08 | 0 | 4 |
| 8 | 18.00 | 4.00 | 29.33 | 9.45 | 0 | 6 |
| 9 | 30.00 | 2.00 | 44.00 | 12.00 | 0 | 12 |
| 10 | 32.00 | 2.00 | 48.00 | 11.14 | 2 | 20 |
| 11 | 38.00 | 6.00 | 53.33 | 14.19 | 2 | 22 |
| 12 | 43.33 | 6.11 | 56.00 | 13.11 | 2 | 32 |
| 13 | 44.67 | 6.43 | 58.00 | 14.00 | 2 | 40 |
| 14 | 48.00 | 5.29 | 60.67 | 15.28 | 2 | 40 |
| 15 | 50.67 | 7.02 | 64.67 | 14.05 | 2 | 44 |
| 16 | 52.00 | 7.21 | 65.33 | 13.01 | 2 | 46 |
| 17 | 52.67 | 6.43 | 67.33 | 12.06 | 2 | 48 |
| 18 | 54.00 | 7.21 | 69.33 | 13.01 | 2 | 48 |
| 19 | 54.67 | 7.02 | 70.67 | 11.02 | 2 | 48 |
| 20 | 55.33 | 8.08 | 71.33 | 11.02 | 2 | 48 |
| 21 | 55.33 | 8.08 | 71.33 | 11.02 | 2 | 48 |
| 22 | 55.33 | 8.08 | 71.33 | 11.02 | 4 | 50 |
| 23 | 55.33 | 8.08 | 71.33 | 11.02 | 6 | 50 |
| 24 | 55.33 | 8.08 | 71.33 | 11.02 | 6 | 50 |
| 25 | 55.33 | 8.08 | 72.00 | 12.00 | 6 | 50 |
| 26 | 55.33 | 8.08 | 72.67 | 11.02 | 6 | 50 |

Treat 8 represents Enteric-coated Oral PEI/pIHNV-G non-pariel sugar beads vaccine.
Treat 9 represents Enteric-coated Oral PEI non-pariel sugar beads vaccine.
Treat 10 represents pIHNV-G intramuscular injection.
Treat 11 represents PBS intramuscular injection.

Statistical analysis suggested no significant difference in the number of mortalities between the individual tanks ($P > 0.05$, chi square test, Table 4.23), thus tank observations were disregarded and treatment groups of 150 fish per group were evaluated.

Similar to the previously performed *in vivo* experiment using enteric-coated spray-dried microparticles vaccine, the symptoms of IHNV, darkened body coloration, vertical drifting, distended abdomens, and pop eye were observed in the dead fish in this study (Figure 4.16). Plaque assay of 10% of the total mortalities confirmed death was caused by IHNV (Figure 4.17).

**Table 4.23: Mortality of fish after challenge with IHNV**

| Treatment[a] | Total number of fish per group | Number of dead fish | | | Total Number of dead fish | Average percent mortality | p-value |
|---|---|---|---|---|---|---|---|
| | | Tank1 | Tank2 | Tank3 | | | |
| 8 | 150 | 24 | 27 | 32 | 83 | 55.33% | 0.267 |
| 9 | 150 | 36 | 42 | 31 | 109 | 72.67% | 0.047 |
| 10 | 50 | 3 | - | - | 3 | 6% | - |
| 11 | 50 | 25 | - | - | 25 | 50% | - |

[a]Treatment 8 represents Enteric-coated Oral PEI/pIHNV-G non-pariel sugar beads vaccine.
Treatment 9 represents Enteric-coated Oral PEI non-pariel sugar beads vaccine.
Treatment 10 represents pIHNV-G intramuscular injection.
Treatment 11 represents PBS intramuscular injection.

# CONCLUSION

The optimum virus concentration needed for the lethal challenge experiment is $10^5$ pfu IHNV per ml for fish immunized with spray-dried enteric protected microparticles and $10^4$ pfu IHNV per ml for those immunized with spray-coated enteric protected beads. Fish immunized by IM injection showed signs of discoloration and swelling at the injection site within the first 24 hours after vaccination. Classic symptoms of IHNV such as darkened body discoloration, vertical drifting, distended abdomens, and pop eye were observed in the dead fish after lethal viral challenge. Plaque assay on approximately 10% of the total mortalities revealed death was caused by IHNV infection. The luciferase activity in the homogenized digestive tract samples was unable to be detected by the luciferase assay system using luminometer. Histological analysis showed no abnormal changes in the fish intestinal tissue samples except for those occasional incidental changes in a few of the specimens.

The high mortality (> 80%) in fish vaccinated orally with spray-dried microparticles containing either liposome/DNA complexes or PEI/DNA complexes revealed that an immune response against IHNV in Rainbow trout could not be induced by this delivery system.

When fish were immunized with spray-coated enteric protected beads embedded with DNA vaccine, a significant difference was observed between fish vaccinated with enteric protected oral vaccine treatment to blank oral treatment, (p-

value < 0.002, chi square test), suggesting an improved level of protection against IHNV by this delivery system compared to oral vaccine microparticles. The efficacy of the spray-coated enteric protected beads oral delivery system in inducing an immune response, however, was not as effective as intramuscular vaccine injection. The mortalities in treatments 8 through 11 was found to be 55.33%, 72.67%, 6%, and 50%, respectively.

The protection efficacy in fish immunized by spray-coated enteric protected beads however, cannot be directly compared to the efficacy of fish immunized by spray-dried microparticles because the experiments were performed separately with different virus strains and concentrations. Further experiments are needed to compare the efficacy of spray-dried enteric protected vaccine microparticles and spray-coated enteric protected beads vaccine delivery systems in inducing an immune response against IHNV. In addition, more research in finding effective vector carriers for DNA vaccines to facilitate their membrane uptake and delivery systems that can prevent the denaturation of plasmid DNA is needed in order to develop a safe, effective, and commercially viable vaccine to control future outbreaks of IHNV.

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# Chapter 5

## Prediction of *in vitro* Drug Release from Matrix Tablets by Applying Statistical and Mathematical Tools

**Angkana Tantituvanont, Jan-Shiang Taur, J. Mark Christensen, Vipaporn Rakkanka, and James W. Ayres**

## ABSTRACT

The purpose of this study was a preliminary investigation of statistically variable amounts of hydrophilic materials, barrier thickness, and paddle speed on drug release characteristics from matrix tablets spray-coated with a barrier membrane designed to produce support platforms in situ. A further purpose was to utilize a mathematical model to predict *in vitro* dissolution profile of cyclobenzaprine hydrochloride from the formulations. Tablets were prepared by direct compression using hydroxypropylmethycellulose (HPMC-E5) and pectin as matrix polymers followed by spray coating with surelease® coating solution. Factorial design was used to determine the effect of two factors: the amount of hydrophilic material in the core tablet and coating amount on drug release from novel matrix tablet spray-coated with barrier membrane. Four test formulations with various amount of HPMC-E5 (12%, 24%, 30%, and 36%) were prepared. Seven levels of coating amount (3%, 6%, 9%, 12%, 15%, and 18%) were employed in each formulation. Dissolution studies were performed in USP apparatus II at paddle speeds of 75, 100, and 125 rpm. Values of exponent n, lag time, and drug release at 6 hours or 10 hours were calculated from each dissolution profile obtained at 75 rpm. These values were used as input variables in multiple linear regressions for model generating. Results showed that as the amount of HPMC/Pectin in the core tablet increased, drug release rate was decreased. Study of barrier thickness as determined by the amount of barrier coating on release characteristics of cyclobenzaprine hydrochloride revealed that, when the coating

amount was increased, drug release rate was decreased along with an increase in lag time. Study of paddle speed effect on release rate of cyclobenzaprine hydrochloride indicated that release rate was often increased as paddle speed increased. Mathematical equations generated from regression analysis of n-value, lag time, and percent drug release as a function of the amount of hydrophilic material and coating amount after accounting for the time period effect could be used in product development as a model for predicting and optimizing *in vitro* drug release of cyclobenzaprine hydrochloride from matrix tablets spray-coated with a barrier membrane. The newly developed equations are:

N-value = $0.56125 + 0.01101\ hpmc + 0.09333\ coat - 0.00330\ coat^2$

Lag time = $-1.14225 + 0.1018\ hpmc + 0.1353\ coat + 0.002480\ (hpmc \times coat) - 0.0021\ hpmc^2$

DR6hr = $90.0855 - 0.7380\ hpmc - 6.3939\ coat + 0.1565\ coat^2$

DR10hr = $91.7716 - 0.7586\ hpmc - 0.1399\ coat^2$

# INTRODUCTION

Development of new pharmaceutical products nowadays includes more emphasize on the pharmacoeconomic value of the product to the consumer since the economic cost and benefit of new products are often taken into consideration in acceptance of these new products by the marketplace (Gardner, 2000). Products were considered to have pharmacoeconomic value when a product produces actual cost savings as well as when a higher cost product is justified by its additional benefits (Gardner, 2000). Controlled release dosage forms have been considered to provide pharmacoeconomic value through increasing clinical effectiveness and reduction in total treatment cost (Gardner, 2000). Several approaches have been used to develop oral drug delivery systems in which the amount of drug release can be controlled to occur at a desired rate. Hydrophilic matrix tablets and membrane coating on drug-loaded beads are two common approaches for extending drug release. Membrane coating typically controls drug release by diffusion of drug through the diffusion membrane (Figure 5.1).

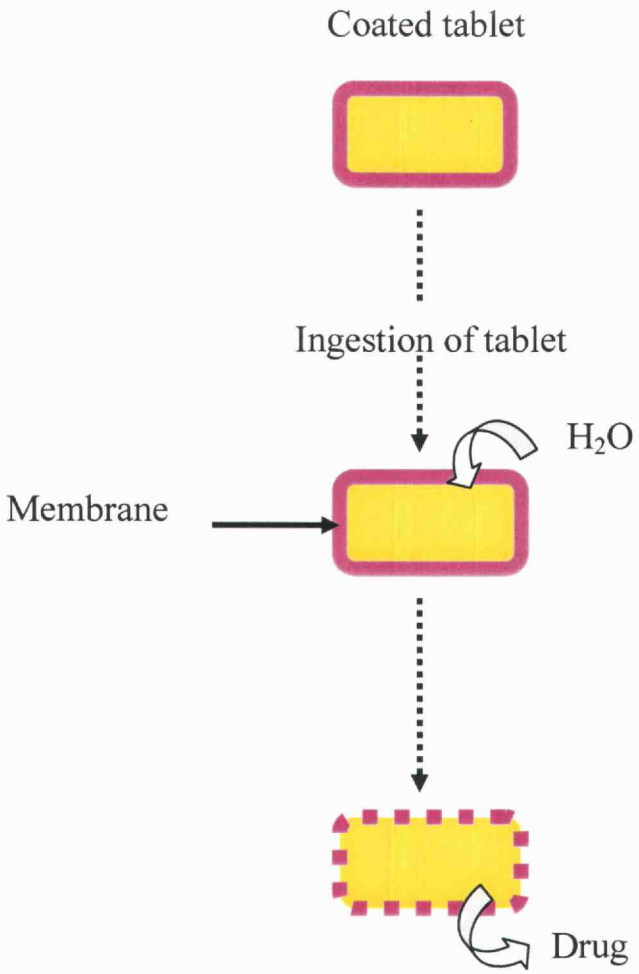


**Figure 5.1: Schematic diagram of membrane coating release.**

Fick's first law has been used to describe release kinetics related with barrier diffusion through membranes (equation 1).

$$\frac{dM}{dt} = ADmP\frac{C_1 - C_2}{X} \qquad \text{equation (1)}$$

$\frac{dM}{dt}$ = Drug release rate

A = Surface area

Dm = Drug diffusion coefficient

P = Polymer/fluid partition coefficient

X = Membrane thickness

$C_1$ = Drug concentration in core tablet

$C_2$ = Drug concentration in fluid adjacent to the surface of the membrane.

In a barrier diffusion system, drug release is strongly dependent on the concentration within the reservoir as the driving force. Assuming that sink conditions exist outside a reservoir, sufficient thickness of membrane coating would create a saturation effect within the reservoir and subsequently constant release of drug (Helms, 1998).

Drug release from hydrophilic matrix tablets is controlled by diffusion of drug through a gelatinous layer formed by hydration of polymer, and erosion of gel (Figure 5.2).

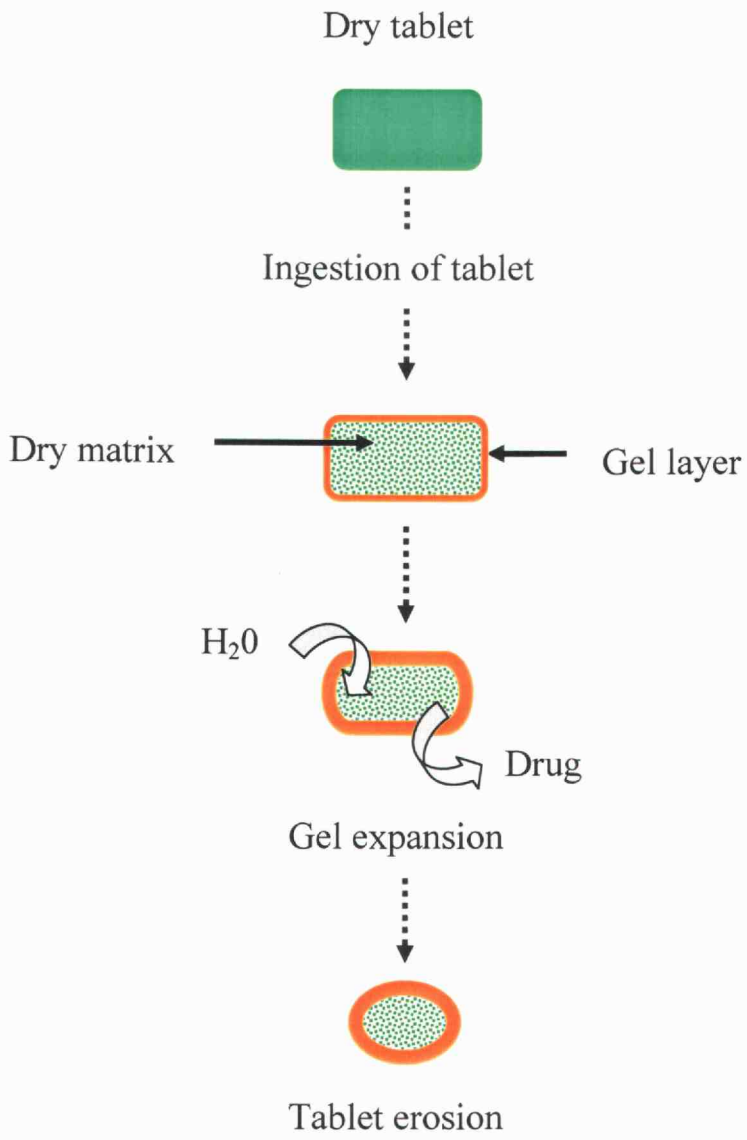


**Figure 5.2: Schematic diagram of hydrophilic matrix release.**

Among a variety of swellable polymers, hydroxypropylmethylcellulose (HPMC), a hydrophilic cellulose derivative, is commonly used for controlling release of drug from matrices due to its rapid hydration, good compression and gelling characteristics being, nontoxic, and ease of use (Khanvilkar et al., 2002; Yan et al., 2000). In the case of water insoluble drug or low viscosity grade of HPMC as rate controlling polymer, overall drug release is highly dependent on surface erosion (Reynolds et al., 2002). For water soluble drugs, diffusional drug release may make the major contribution to overall drug release from matrix tablets (Reynolds et al., 2002). Drug release profile of water soluble drug through the matrices generally follows the square root of time relationship established by Higuchi (equation 2) (Ford et al., 1985; Helms, 1998; Khanvilkar et al., 2002). Higuchi's equation, however, fails to consider the change in volume upon swelling of the matrix and erosion of the matrix by hydration.

$$\frac{Wr}{t^{1/2}} = 2W_0\left(\frac{S}{V}\right)\left(\frac{D^1}{\pi}\right)^{1/2} \qquad \text{equation (2)}$$

Wr = Amount of drug released at time t

Wo = Dose of the drug

S = Diffusional area

V = Volume of hydrated matrix

$D^1$ = Diffusion coefficient of the drug in the matrix

In the present research, an equation derived by Korsmeyer and Peppas (equation 3) was employed to describe the drug release mechanism from a hydrophilic swellable matrix tablet. This equation was further modified by Ford et al. in order to account for the lag time (Helms, 1998) (equation 4). The exponent n calculated from the modified equation (equation 4) was used to determine release kinetics in a new combined hydrophilic matrix tablet with an applied barrier diffusion system.

$$\frac{Mt}{M_\infty} = kt^n \qquad \text{equation (3)}$$

$\frac{Mt}{M_\infty}$ = Fraction of drug release at time t

k = Kinetic constant

t = Release time

n = Diffusional exponent of drug release

$$\frac{M(t-l)}{M_\infty} = k(t-l)^n \qquad \text{equation (4)}$$

l = lag time

Hydrophilic matrix tablets and membrane coating technologies have been combined at Oregon State University to produce membrane coated hydrophilic matrix tablets that swell in the gastrointestinal tract to rupture the membrane coating in the belly-band area of the tablet and retain platform supports on lateral surfaces of the tablets (Figure 5.3).

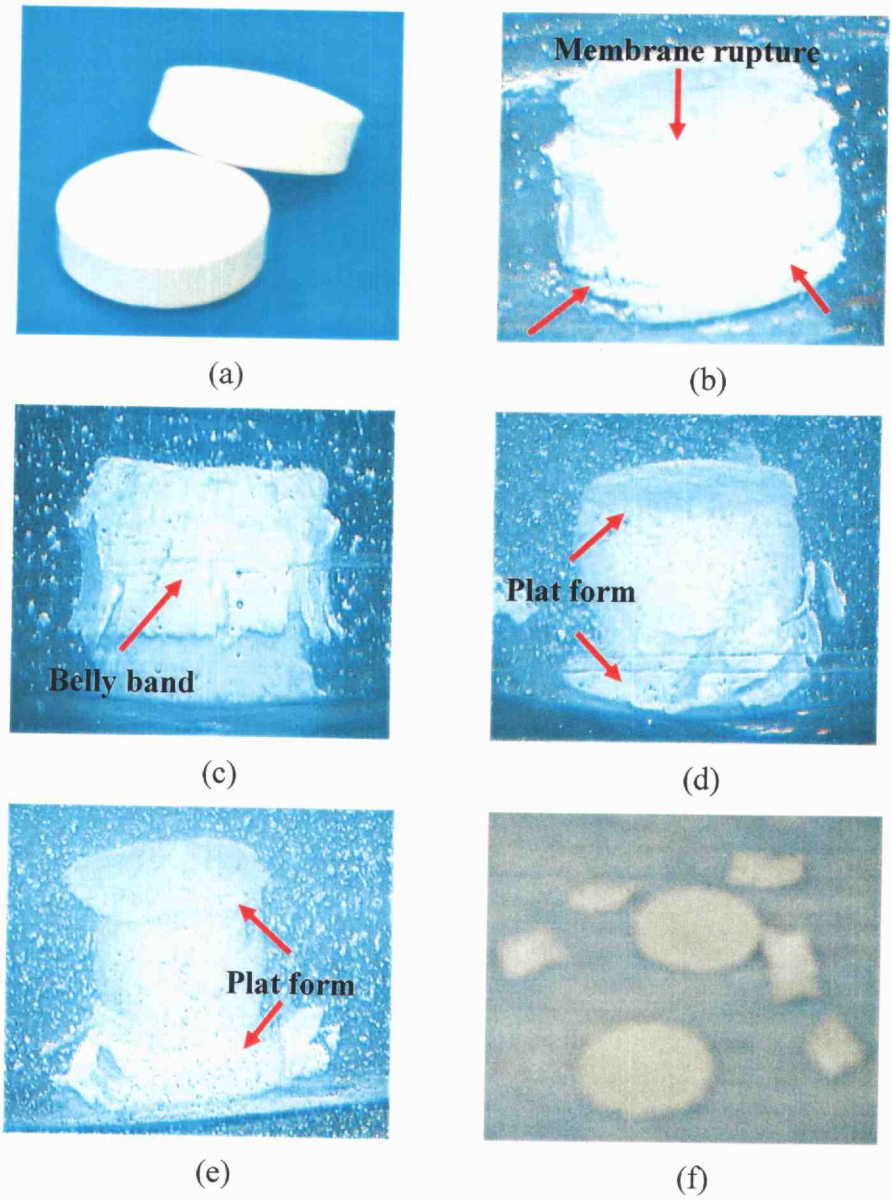


**Figure 5.3: Drug release from matrix tablet spray coated with membrane.**
(a) Dry tablet (b-c) Swelling of matrix tablet in gastrointestinal fluids and rupture of membrane in the belly band area of the tablet (d-f) Retention of platform support membranes on the lateral surface of tablet.

Controlling formulation parameters such as hydrophilic matrix polymer blends and amounts along with support platform generating membrane characteristics and tablet shape allow development of controlled release tablets with an almost infinite variety of drug release patterns that can be relatively insensitive to gastrointestinal activity and pH. Thus, among other possibilities, these formulations can mimic drug release from osmotic pump tablets, if desired.

In new formulation development, a large amount of actual physical experimentation is conducted based on trial and error. This often lengthens processing time and depends on the experience of researchers. Applying statistical and mathematical tools or computer software such as artificial neuro networks (ANNs) (Peh et al., 2000) can help solve problems regarding process optimization and product development, resulting in cost effectives in terms of time and man power. Statistical and mathematical approaches are useful in evaluating relationships of variables in the data and generating model equations that relate input and output variables of the question of interest. In this study, statistical procedures were used to investigate relationships between drug release characteristics and amount of hydrophilic material in a core tablet, along with variable barrier thickness. This relationship was further used to generate mathematical model equations that allow researchers to predict release characteristics of drug at different amounts of hydrophilic material and barrier thickness. Additionally, dosage formulations can be optimized and predicted based on mathematical equations that characterize dissolution profiles.

The purpose of this study was a preliminary investigation to evaluate statistically variable amounts of hydrophilic materials, barrier thickness, and paddle speed on the drug release characteristics and to utilize mathematical model to predict and optimize *in vitro* cyclobenzaprine hydrochloride release from matrix tablets spray-coated with a barrier membrane designed to produce support platforms in situ.

## MATERIALS AND METHODS

Cyclobenzaprine HCL as a model drug, dicalcium phosphate anhydrous powder as a diluent, and opadry II as a component of coating membrane were a gift from Teva pharmaceuticals (Sellersville, PA, USA). Methocel E5 (hydroxypropylmethylcellulose) viscosity of 4-6 cps and pectin USP as hydrophilic materials was purchased from Spectrum, Spectrum Quality Products, Inc. (Gardena, CA, USA). Stearic acid USP (Triple pressed) as a lubricant was obtained from J.T. baker Chemical Co. (Phillipsburg, NJ, USA). Surelease® (ethylcellulose) as a membrane coating was a gift from Colorcon (West Point, PA, USA).

### I. Tablet Formulation

### Experimental Design

Factorial design with four levels of hydrophilic materials (HPMC-E5, 12%, 24%, 30%, and 36%) and seven levels of coating amount (0%, 3%, 6%, 9%, 12%, 15%, and 18%) (Table 5.11) was employed to study the effect of HPMC-E5 amount and barrier thickness on release characteristics of cyclobenzaprine hydrochloride from matrix tablets spray-coated with barrier membrane.

**Matrix Tablet Preparation**

To evaluate the effect of hydrophilic materials (HPMC-E5 and pectin) on cyclobenzaprine hydrochloride release rate, four different levels of HPMC-E5 with the constant ratio of 6 to 1 HPMC-E5 to Pectin was studied. Matrix tablets were prepared according to formulations listed in Table 5.1.

**Table 5.1: Formulation for matrix tablet preparation**

| Ingredients (%) | Formulation | | | |
|---|---|---|---|---|
| | MT1 | MT2 | MT3 | MT4 |
| Cyclobenzaprine HCL | 4 | 4 | 4 | 4 |
| HPMC-E5 | 12 | 24 | 30 | 36 |
| Pectin | 2 | 4 | 5 | 6 |
| Dicalcium phosphate anhydrous | 79.5 | 65.5 | 58.8 | 51.5 |
| Stearic acid | 2.5 | 2.5 | 2.5 | 2.5 |

Briefly, Cyclobenzaprine hydrochloride and inactive ingredients except stearic acid were mixed by shaking in a brown glass bottle in appropriate amounts depending on batch size for 15 minutes. After 15 minutes, stearic acid was added into the mixture of powder followed by an additional 5 minutes mixing prior to compression. The mixture was then compressed into round, biconvex tablets (11.15 mm diameter) at a pressure of 5000 lbs using a Carver press fitted with punches and a die , FRED' S CARVER, Inc., Hydraulic equipment summit (NJ, USA). Dwell time after the target

pressure was achieved was 10 seconds and each tablet weight was 750 mg. Fifty tablets of each formula were coated with surelease®/opadry solution (Table 5.2) using Niro Fluid Bed Laboratory Unit STEREA-1, Niro Inc., Aeromatic-Fielder Div. (Columbia, MD, USA).

**Table 5.2: Formula for surelease® coating solution**

| Ingredients | Amount |
|---|---|
| Surelease® | 64 ml |
| Opadry | 10.125 g |
| Talc | 21.307 g |
| Deionized water | 300 ml |

Coating parameters were controlled such that all tablets were prepared under identical conditions (Table 5.3). Tablets were sampled at 0%, 3%, 6%, 9%, 12%, 15%, and 18% theoretical weight gain for determining the effect of barrier amount on release characteristics of cyclobenzaprine HCL.

**Table 5.3: Coating parameters**

| **Parameters** | |
|---|---|
| Solution feed rate | 2.8 ml/min |
| Inlet air temperature | 60°C |
| Outlet air temperature | 55°C |
| Atomized air pressure | 15 psi |
| Fluidized air | 85 % of full capacity |
| Nozzle size | 0.8 mm |
| Drying time: | 5 minutes |

### *In vitro* Release of Cyclobenzaprine HCL

Release rate of cyclobenzaprine hydrochloride from the matrix tablets was investigated using USP dissolution apparatus II, Vankel Industries, Inc. (Edison, NJ, USA). Dissolution testing was performed in triplicate with 900 ml simulated intestinal fluid pH 7.4. Paddle speed was set as defined in different experiments with bath temperature set at 37.5 °C. To evaluate the effect of paddle speed on drug release characteristics, different paddle speeds of 75, 100, and 125 rpm were utilized. Samples of 3 ml were taken at times, 5, 10, 15, 30, 45 minutes, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 hours and filtered through a 70 micron filter. Filtrate was then centrifuged at 3000 rpm (956 ×g) for 5 minutes followed by UV spectrophotometer analysis at wavelength 289 nm using a Beckman spectrophotometer, DU Series 600, Beckman Instruments, Inc. (Fullerton, CA, USA).

## II. Strategy for Data Analysis

Statistical analysis based on multiple linear regression was applied to investigate the relationship of hydrophilic materials (HPMC E-5) and barrier thickness on drug release of cyclobenzaprine hydrochloride from matrix tablets. A set of tablets prepared according to MT1, MT2, and MT4 formulations was used in model generation; another set of tablets prepared according to MT3 formulation was used in model validation (Diagram 5.1). The analysis was performed with the SAS system, SAS Institute Inc. (Cary, NC, USA).

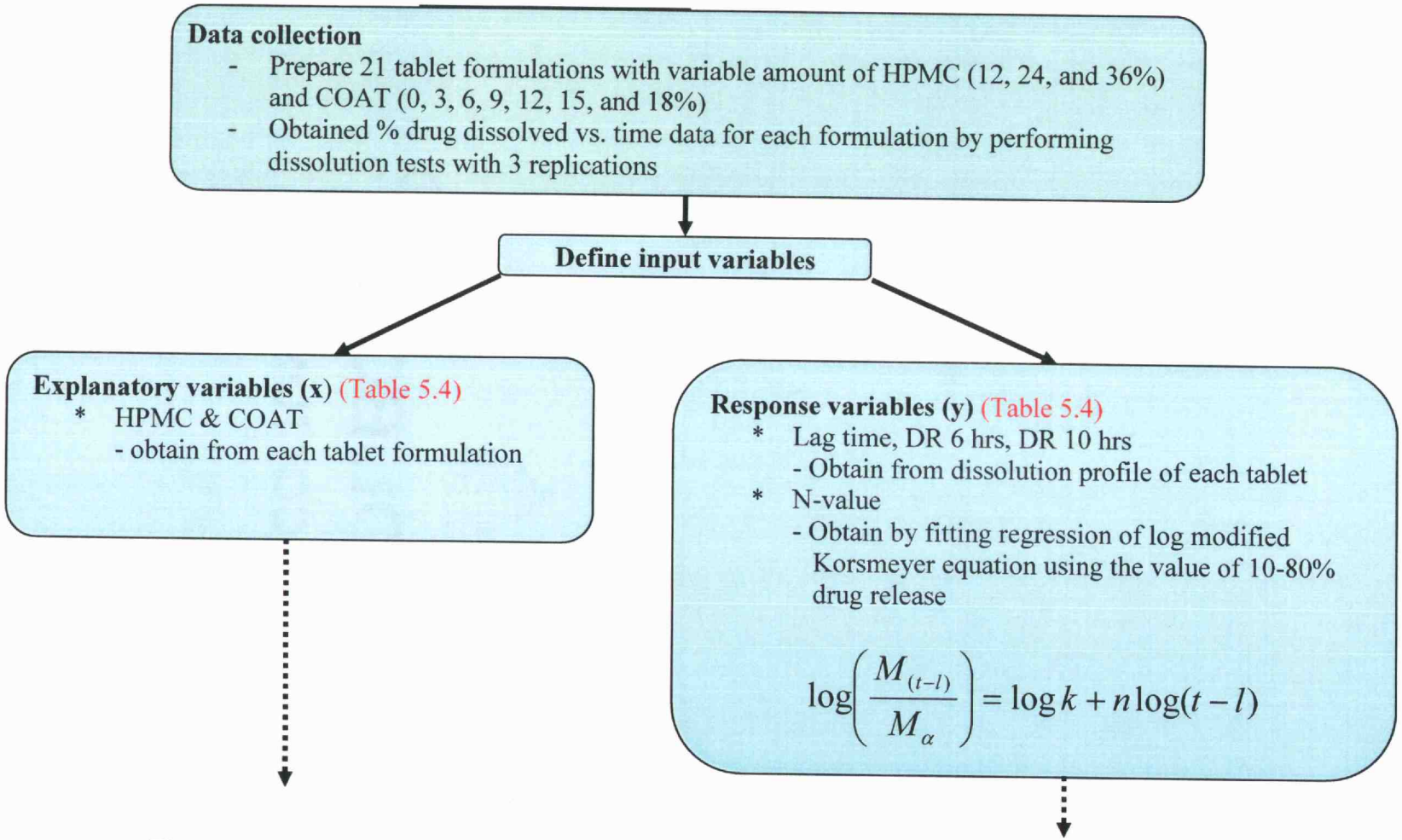


**Figure 5.3a: A Strategy for Data Analysis using Statistical Models (Ramsey and Schafer, 1997).**

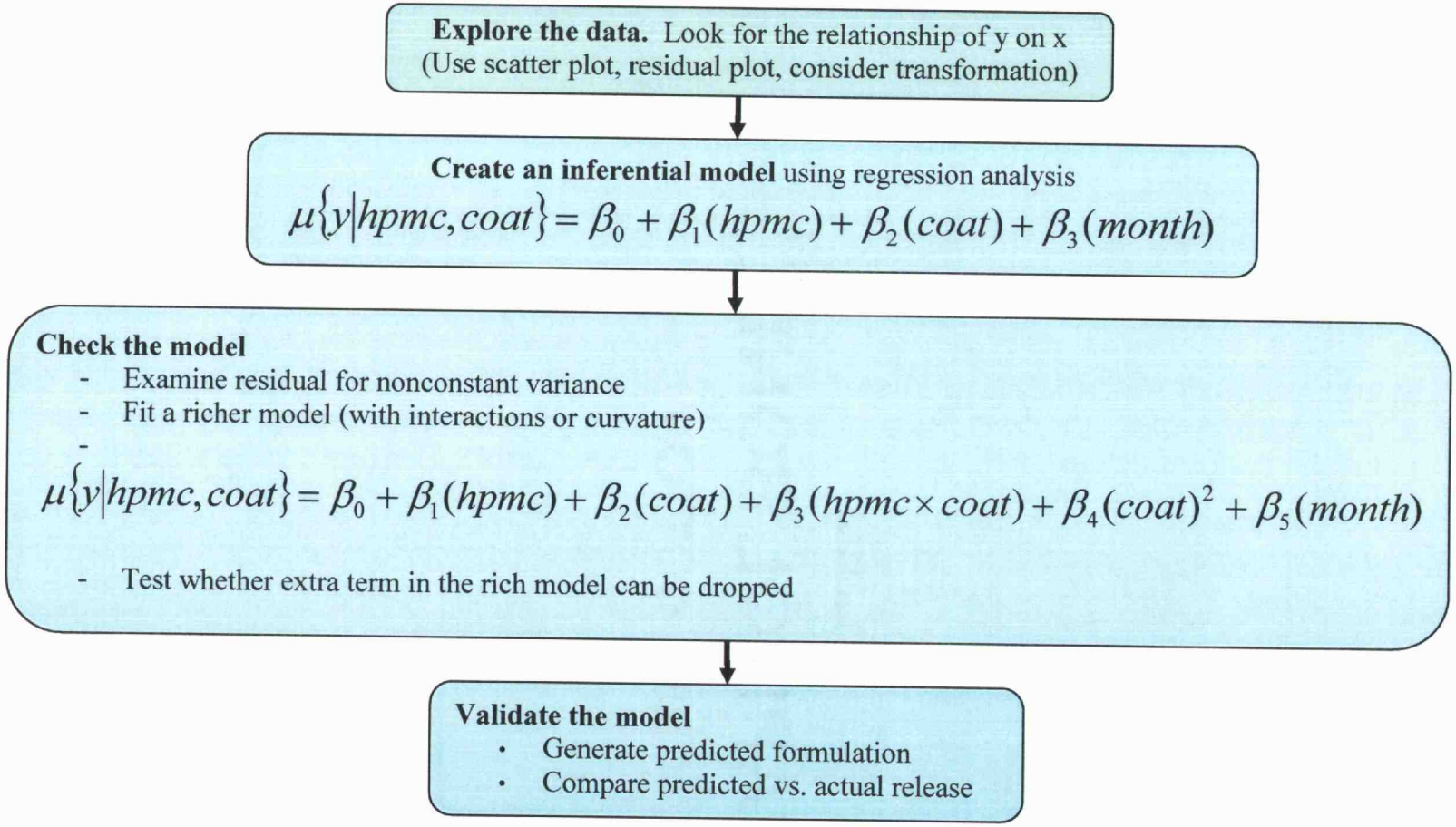


**Figure 5.3a: A Strategy for Data Analysis using Statistical Models (continued)(Ramsey and Schafer, 1997).**

**Table 5.4: Calculated n-value, lag time, percent drug release at 6 hours and 10 hours**

| % HPMC in core tablet | % Coating | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0% | 3% | 6% | 9% | 12% | 15% | 18% |
| | 12% | % DR 6 HRS[a] | 83.4<br>72.5<br>59.6 | 68.8<br>68.9<br>70.7 | 52.3<br>54.0<br>43.2 | 33.1<br>34.8<br>35.8 | 29.0<br>33.1<br>29.8 | | |
| | | Mean | 71.83 | 69.47 | 49.83 | 34.57 | 30.63 | | |
| | | SD | 11.91 | 1.07 | 5.81 | 1.36 | 2.17 | | |
| | | % DR 10 HRS[b] | | | 68.4<br>73.7<br>60.5 | 72.7<br>73.0<br>71.8 | 57.4<br>62.1<br>60.2 | 63.9<br>68.0<br>63.0 | 33.2<br>32.9<br>40.3 |
| | | Mean | | | 67.53 | 72.5 | 59.9 | 64.97 | 35.47 |
| | | SD | | | 6.64 | 0.62 | 2.36 | 2.67 | 4.19 |
| | | N-value | 0.41<br>0.42<br>0.45 | 1.02<br>1.02<br>1.135 | 0.91<br>0.95<br>0.77 | 1.42<br>1.36<br>1.40 | 1.19<br>1.48<br>1.42 | 1.78<br>1.32<br>1.46 | 1.16<br>1.01<br>1.16 |
| | | Mean | 0.43 | 1.06 | 0.88 | 1.39 | 1.36 | 1.52 | 1.11 |
| | | SD | 0.02 | 0.07 | 0.09 | 0.03 | 0.15 | 0.24 | 0.09 |
| | | Lag time | 0<br>0<br>0 | 0.25<br>0.25<br>0.25 | 1<br>1<br>1 | 1.25<br>1.25<br>1.25 | 1.5<br>1.5<br>1.5 | 2<br>2<br>2 | 3<br>3<br>3 |
| | | Mean | 0 | 0.25 | 1 | 1.25 | 1.5 | 2 | 3 |
| | | SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a]Percent drug release at 6 hours
[b]Percent drug release at 10 hours

**Table 5.4: Calculated n-value, lag time, percent drug release at 6 hours and 10 hours (continued).**

| %.HPMC in core tablet | % Coating | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0% | 3% | 6% | 9% | 12% | 15% | 18% |
| | 24% | % DR 6 HRS[a] | 90.3<br>83.4<br>78.7 | 53.8<br>50.8<br>60.0 | 37.3<br>30.8<br>33.2 | 28.3<br>30.2<br>21.7 | 10.8<br>8.0<br>18.8 | | |
| | | Mean | 84.13 | 54.87 | 33.77 | 26.73 | 12.53 | | |
| | | SD | 5.83 | 4.69 | 3.29 | 4.46 | 5.60 | | |
| | | % DR 10 HRS[b] | | | 70.0<br>60.7<br>65.0 | 71.0<br>70.1<br>63.5 | 43.72<br>41.12<br>58.40 | 40.45<br>41.51<br>42.14 | 19.68<br>20.09<br>12.97 |
| | | Mean | | | 65.23 | 68.2 | 47.75 | 41.37 | 17.58 |
| | | SD | | | 4.65 | 4.09 | 9.32 | 0.85 | 4.00 |
| | | N-value | 0.77<br>0.79<br>0.77 | 1.29<br>1.25<br>1.28 | 1.05<br>1.23<br>1.29 | 1.37<br>1.37<br>1.31 | 1.65<br>1.63<br>1.50 | 1.42<br>1.77<br>1.70 | 1.40<br>1.21<br>1.40 |
| | | Mean | 0.78 | 1.27 | 1.19 | 1.35 | 1.59 | 1.63 | 1.34 |
| | | SD | 0.01 | 0.02 | 0.12 | 0.03 | 0.08 | 0.18 | 0.11 |
| | | Lag time | 0<br>0<br>0 | 0.45<br>0.45<br>0.45 | 1.5<br>1.5<br>1.5 | 1.75<br>1.75<br>1.75 | 2.5<br>2.5<br>2.5 | 3<br>3<br>3 | 4<br>4<br>4 |
| | | Mean | 0 | 0.45 | 1.5 | 1.75 | 2.5 | 3 | 4 |
| | | SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a]Percent drug release at 6 hours
[b]Percent drug release at 10 hours

**Table 5.4: Calculated n-value, lag time, percent drug release at 6 hours and 10 hours (continued).**

| % HPMC in core tablet | % Coating | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0% | 3% | 6% | 9% | 12% | 15% | 18% |
| | 36% | % DR 6 HRS[a] | 71.10<br>49.80<br>60.45 | 24.12<br>46.37<br>56.21 | 30.93<br>29.70<br>30.31 | 16.62<br>16.79<br>40.79 | 8.56<br>9.76<br>12.54 | | |
| | | Mean | 60.45 | 42.23 | 30.31 | 24.73 | 10.29 | | |
| | | SD | 10.65 | 16.44 | 0.62 | 13.91 | 2.04 | | |
| | | % DR 10 HRS[b] | | | 62.73<br>63.74<br>64.74 | 49.70<br>53.22<br>74.26 | 25.53<br>34.49<br>37.70 | 30.57<br>36.13<br>33.32 | 22.66<br>16.42<br>23.35 |
| | | Mean | | | 63.74 | 59.06 | 32.57 | 33.34 | 20.81 |
| | | SD | | | 1.01 | 13.28 | 6.31 | 2.78 | 3.82 |
| | | N-value | 0.88<br>0.85<br>0.84 | 1.73<br>1.39<br>1.28 | 1.26<br>1.43<br>1.35 | 1.62<br>1.67<br>0.98 | 1.36<br>1.59<br>1.26 | 1.65<br>1.45<br>1.51 | 1.57<br>1.61<br>1.50 |
| | | Mean | 0.86 | 1.47 | 1.35 | 1.42 | 1.40 | 1.54 | 1.56 |
| | | SD | 0.02 | 0.23 | 0.08 | 0.38 | 0.17 | 0.10 | 0.06 |
| | | Lag time | 0<br>0<br>0 | 0.5<br>0.5<br>0.5 | 1.5<br>1.5<br>1.5 | 1.75<br>1.75<br>1.75 | 2.5<br>2.5<br>2.5 | 3<br>3<br>3 | 4<br>4<br>4 |
| | | Mean | 0 | 0.5 | 1.5 | 1.75 | 2.5 | 3 | 4 |
| | | SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a]Percent drug release at 6 hours
[b]Percent drug release at 10 hours

**Determination of Input Variables for Statistical Analysis**

Input variables consisted of response variables and explanatory variables. Explanatory variables were percent HPMC-E5 in the core tablet, coating amount, which were controlled by researcher, and time periods. Two different time periods were employed in this study named; time 1 and time 2. Tablet with 0%, 6%, 12%, and 18% coating were prepared at time 1 while tablets with 3%, 9%, and 15% were produced at time 2. Response variables were n-value, lag time, % drug release at 6 hours, and % drug release at 10 hours. Values of response variables were obtained from dissolution profiles at 75 rpm for tablets prepared according to MT1, MT2, and MT4 formulation of Table 5.1. Each formulation was made with seven different coating levels (0%, 3%, 6%, 9%, 12%, 15%, and 18%). Tablet formulations with 0%, 6%, 12%, and 18% coating were made in time 1 while tablet formulation with 3%, 9%, and 15% were made in time 2. The dissolution profile of each formulation was obtained by performing the dissolution test with three replications. An individual value calculated from each dissolution profile was used in the regression analysis rather than its average value. Lag time (time which no drug release) and percent drug release at 6 hours and 10 hours can be obtained directly from the *in vitro* dissolution data for each tablet formulation (Figure 5.4a and 5.4b).

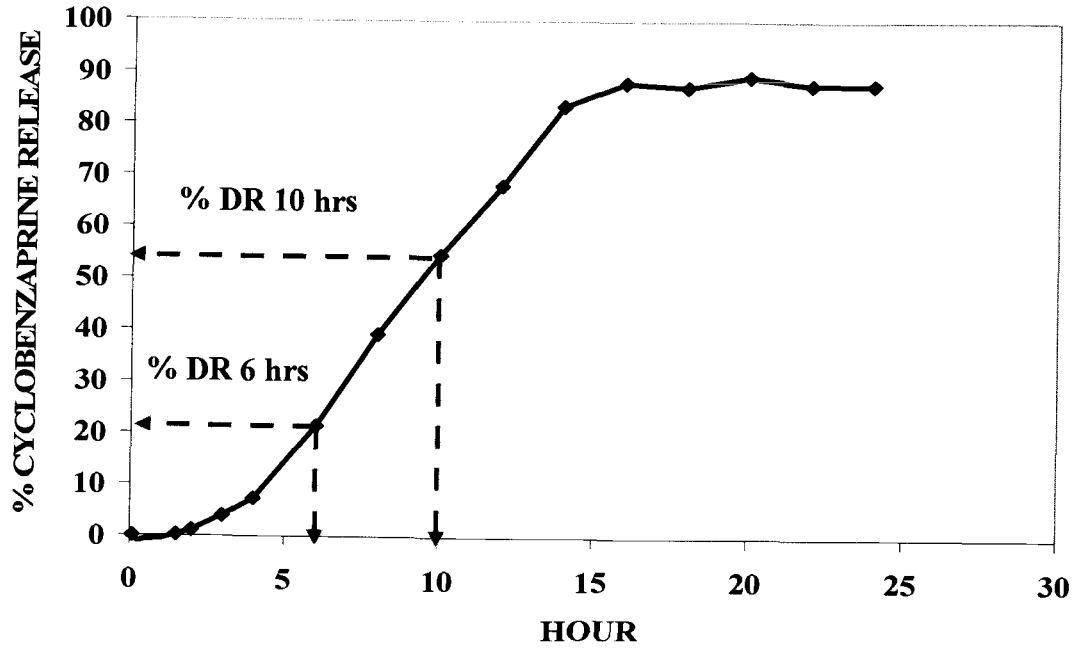


**Figure 5.4a: Calculation of percent drug Release at 6 hours and 10 hours.**

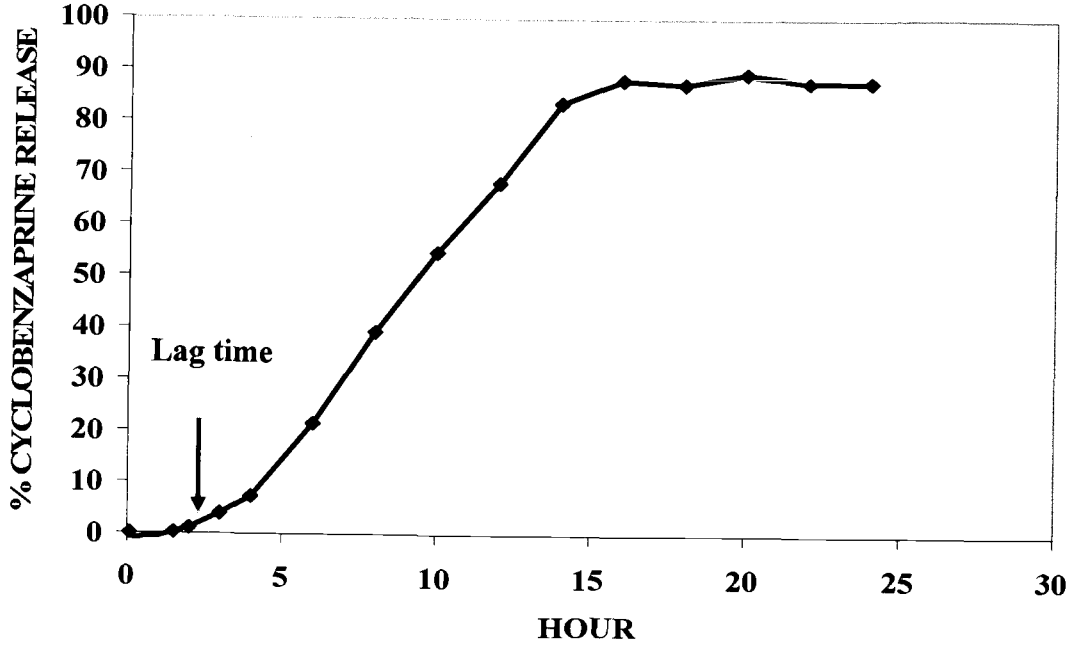


**Figure 5.4b: Calculation of lag time.**

N-value was calculated from log modified Korsmeyer equation (equation 5) using linear regression of values between 10% and 80% drug release (Figure 5.5a-5.5b).

$$\log\left(\frac{M_{(t-l)}}{M_{\alpha}}\right) = \log k + n\log(t-l) \qquad \text{equation (5)}$$

$$y = b + ax$$

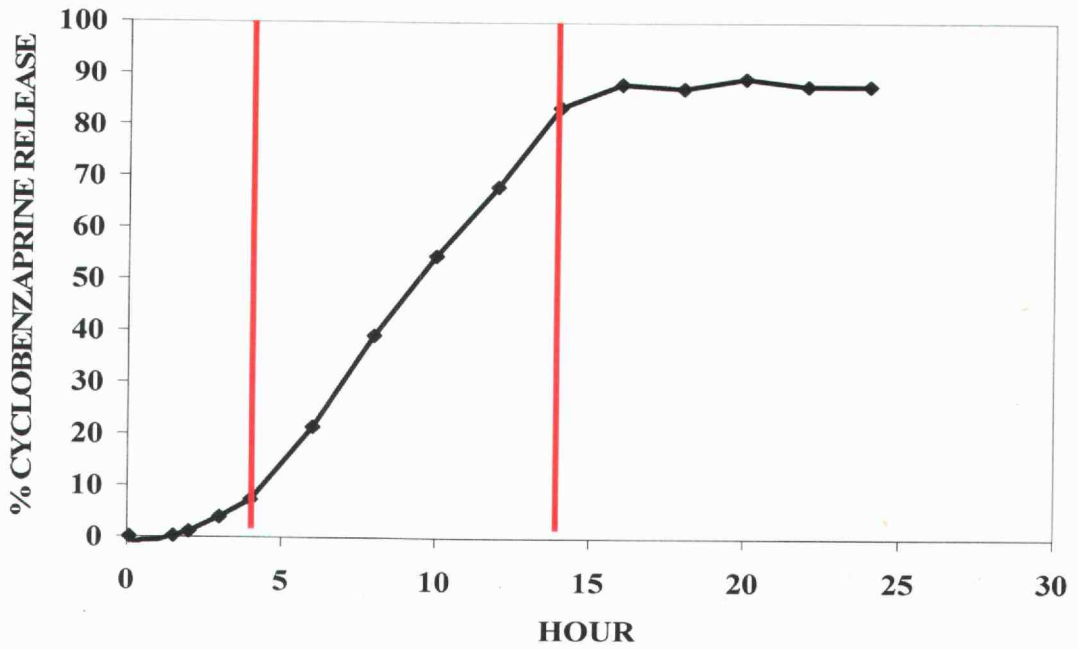


**Figure 5.5a: Calculation of n-value using log modified Korsmeyer equation.**

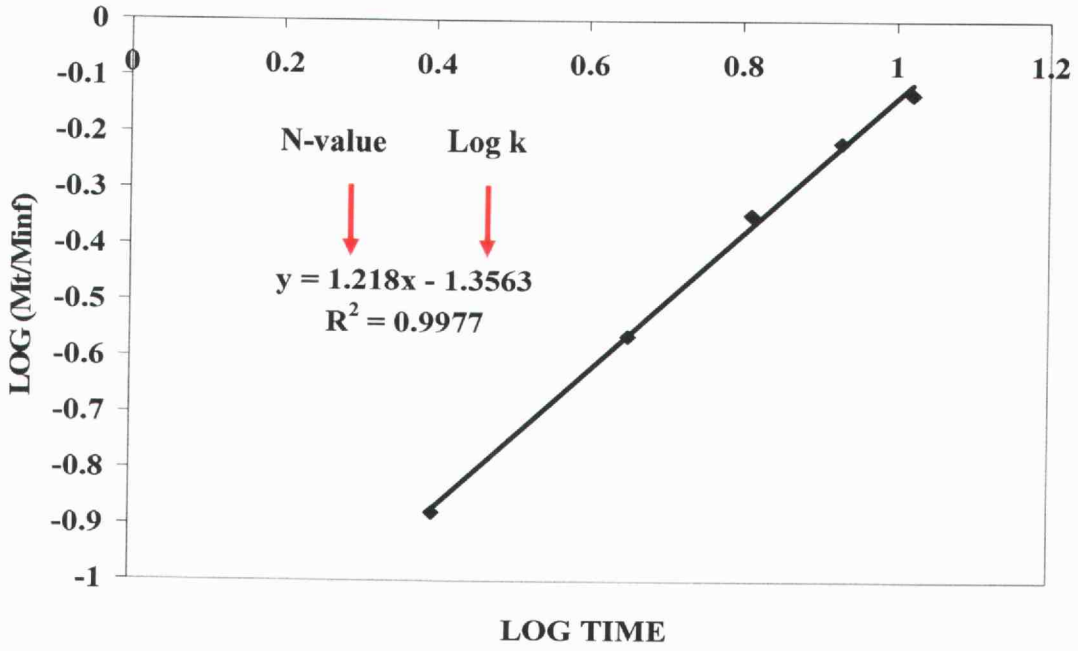


**Figure 5.5b: Linear regression of n-value.**

## Inspection of Input Variables

Graphical tools such as coded scatterplots and residual plots were used for examining the relationship between response variables and explanatory variables, for considering the need of transformation and the addition of extra terms.

## Regression Analysis

Multiple linear regressions were performed using mixed linear model to find the best fit model for predicting percent cyclobenzaprine HCL release from these new matrix tablets. Regression analysis began by fitting the rich model (model with

interactions or curvature) of each response variable as a function of explanatory variables. Typical model equation for multiple linear regressions is presented in equation 6.

$$y = \beta_0 + \beta_1 \chi_1 + \beta_2 \chi_2 + \ldots \beta_n \chi_n \ldots \qquad \text{equation (6)}$$

| | | |
|---|---|---|
| $y$ | = | Response variable |
| $\beta_0$ | = | Constant |
| $\beta_1 \ldots \beta_n$ | = | Model coefficients |
| $\chi_1 \cdots \chi_n$ | = | Level of explanatory variables |

Residual plot obtained from the fit was used for examining nonconstant variance. Validity of coefficients assigned to each explanatory variable was examined using t-test. P-value obtained from the fit was used as an initial indication to consider whether the extra term in the rich model can be dropped. To test whether extra term in the rich model can be dropped, reduced model (model with the drop out term) was fit and the extra sum of square F-test was used to examine the lack of fit. Once the regression analysis, validity checked, and lack of fit test were completed, mathematical model equations were generated for predicting the value of response variables as a function of explanatory variables.

**Validation of Mathematical Models**

Tablets prepared according to MT3 formulation of Table 5.1 with seven levels of coating amount (0%, 3%, 6%, 9%, 12%, 15%, and 18%) were used as test formulations in model validation. The efficiency of the mathematical model was determined by comparing *in vitro* dissolution profiles of these test formulations to their model predicted dissolution profiles.

## RESULTS AND DISCUSSION

### I. Tablet Formulation

The release rate of drug from matrix tablets depends strongly on the diffusion of drug through a gelatinous layer formed by hydrated polymer (HPMC-E5 and pectin in this case), as well as erosion of the gel. To determine the effect of HPMC/Pectin on cyclobenzaprine hydrochloride release rate, dissolution of matrix tablets containing four different amounts of HPMC/Pectin was examined. Results showed that as the amount of HPMC/Pectin in the core tablet increased, drug release rate was decreased which is the expected outcome. Drug release was completed at 5, 8, 12, and 16 hours in formulations containing 12%, 24%, 30%, and 36% HPMC (MT1, MT2, MT3, and MT4) respectively (Figure 5.6).

Study of barrier thickness (as determined by amount of barrier coating) on release characteristics of cyclobenzaprine hydrochloride revealed that, when the coating amount was increased, drug release rate was decreased along with an increase in lag time (Figures 5.7-5.10). In MT1 formulation, lag times of 0.25, 1, 1.25, 1.5, 2, and 3 hours were observed with coating amounts of 3%, 6%, 9%, 12%, 15%, and 18%, respectively (Figure 5.7). MT2, MT3, and MT4 formulations produced lag times of 0.45, 1.5, 1.75, 2.5, 3, and 4 hours with the coating amounts of 3%, 6%, 9%, 12%, 15% and 18%, respectively (Figure 5.8-5.10). Time to complete fifty percent drug release was increased from 4.5 to 12 hours, 5.5 to 17 hours, 6.5 to 16 hours, and

7 to 14 hours when the coating amount was increased from 3% to 18% in MT1, MT2, MT3, and MT4 formulation, respectively (Figures 5.7-5.10). Results from the study of paddle speed effect on release rate of cyclobenzaprine hydrochloride indicated that release rate was usually increased as paddle speed increased (Figure 5.11-5.22). Increasing the amount of matrix polymer helps decrease the effect of paddle stirring speed. Thus, it can be seen in Figure 5.20, for example, that there is relatively little effect of paddle speed.

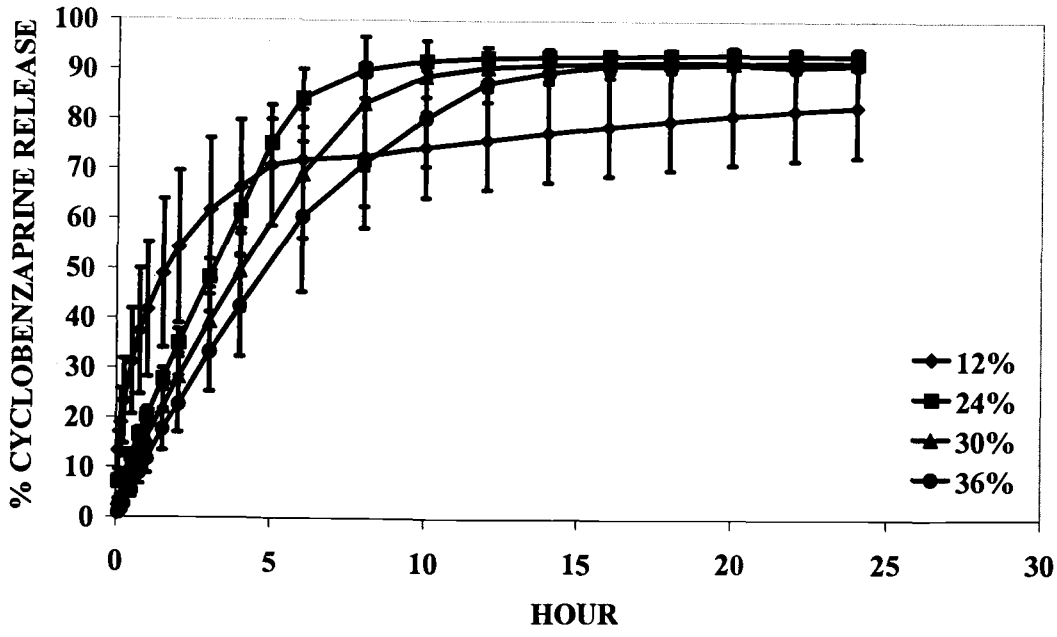


**Figure 5.6: Effect of HPMC variation on release of cyclobenzaprine HCL from uncoated tablets. Mean ± SD (n=3).**

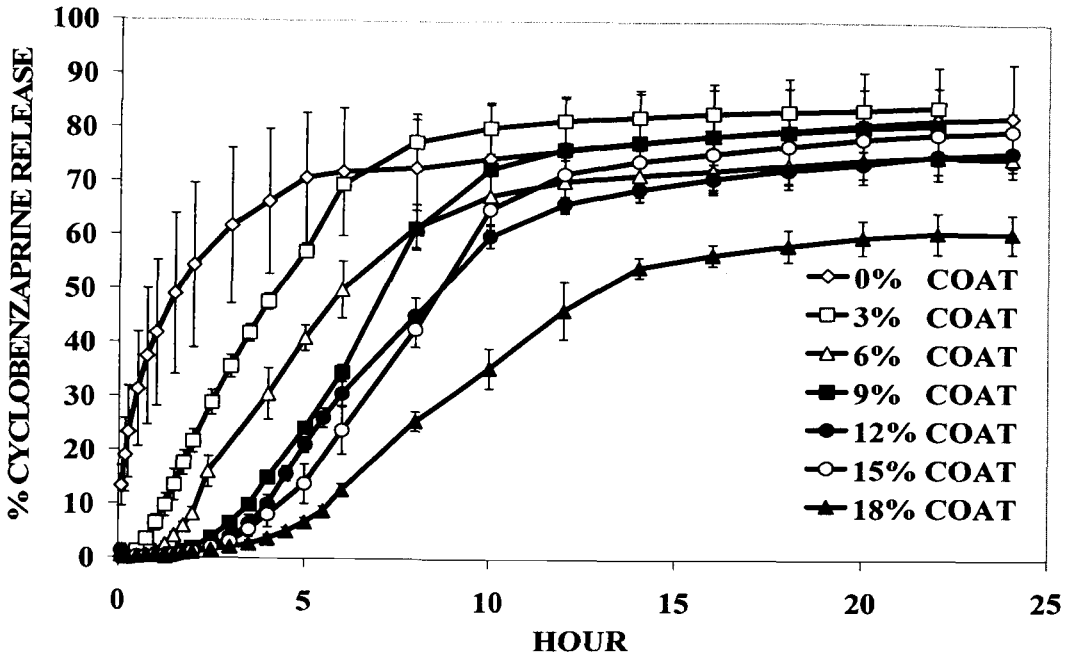


**Figure 5.7: Effect of barrier thickness variation on release of cyclobenzaprine HCL from matrix tablets containing 12% HPMC-E5. Mean ± SD (n=3).**

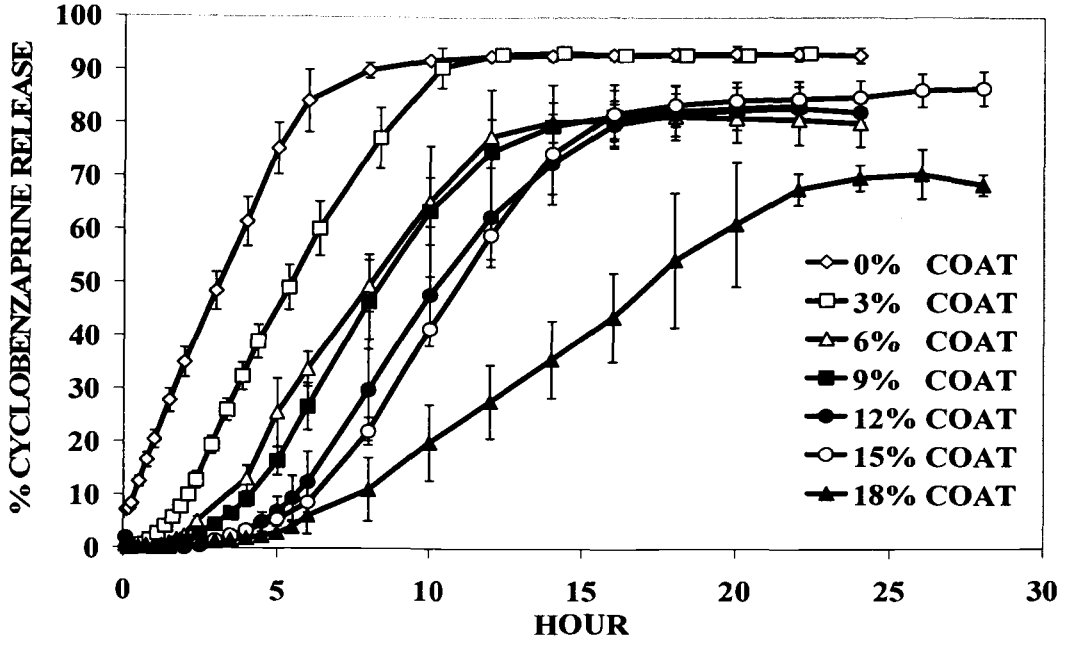


**Figure 5.8: Effect of barrier thickness variation on release of cyclobenzaprine HCL from matrix tablets containing 24% HPMC-E5. Mean ± SD (n=3).**

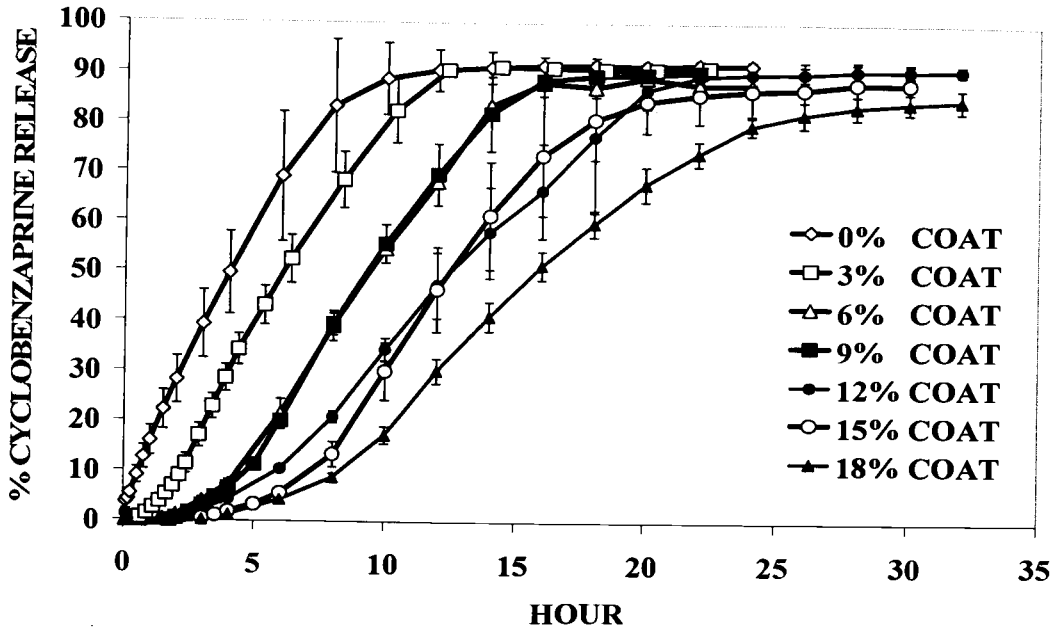


**Figure 5.9: Effect of barrier thickness variation on release of cyclobenzaprine HCL from matrix tablets containing 30% HPMC-E5. Mean ± SD (n=3).**

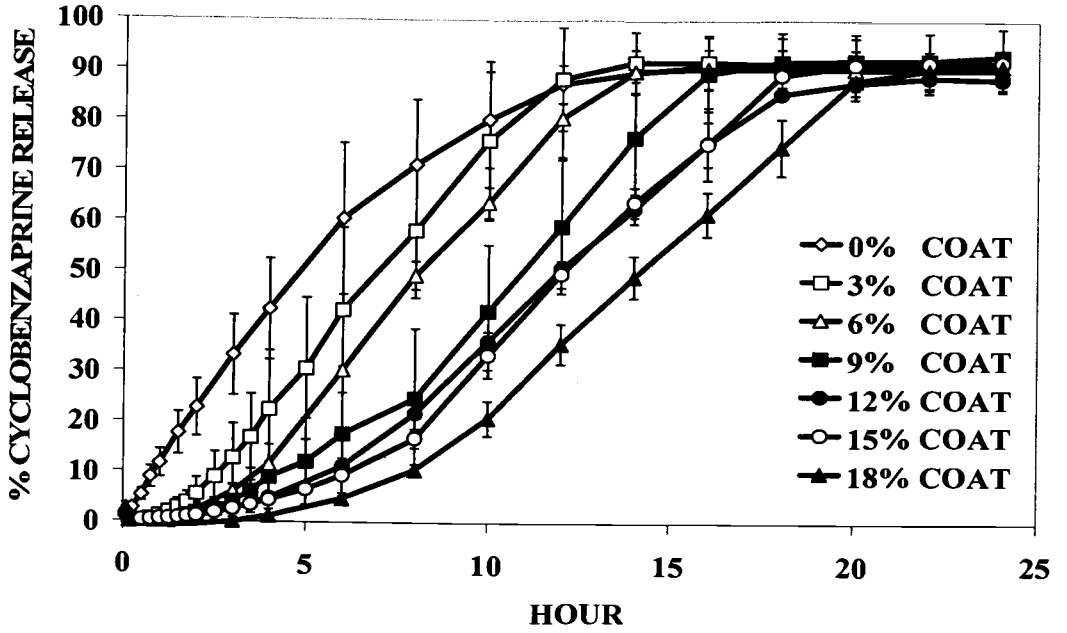


**Figure 5.10: Effect of barrier thickness variation on release of cyclobenzaprine HCL from matrix tablets containing 36% HPMC-E5. Mean ± SD (n=3).**

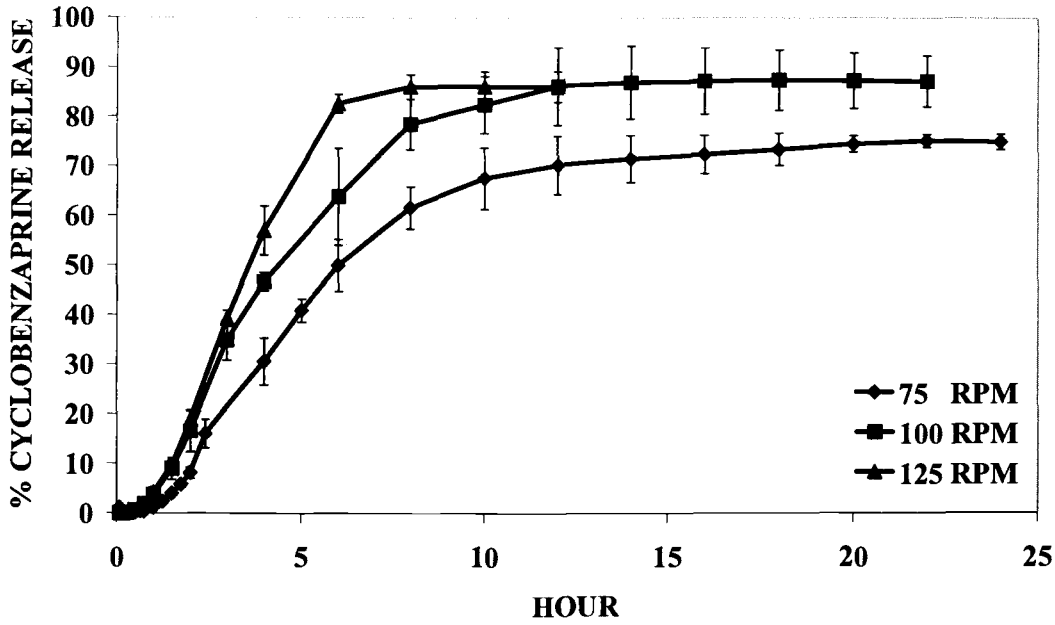


**Figure 5.11: Effect of paddle speed on release of cyclobenzaprine HCL from matrix tablets containing 12% HPMC-E5 and 6% coating. Mean ± SD (n=3).**

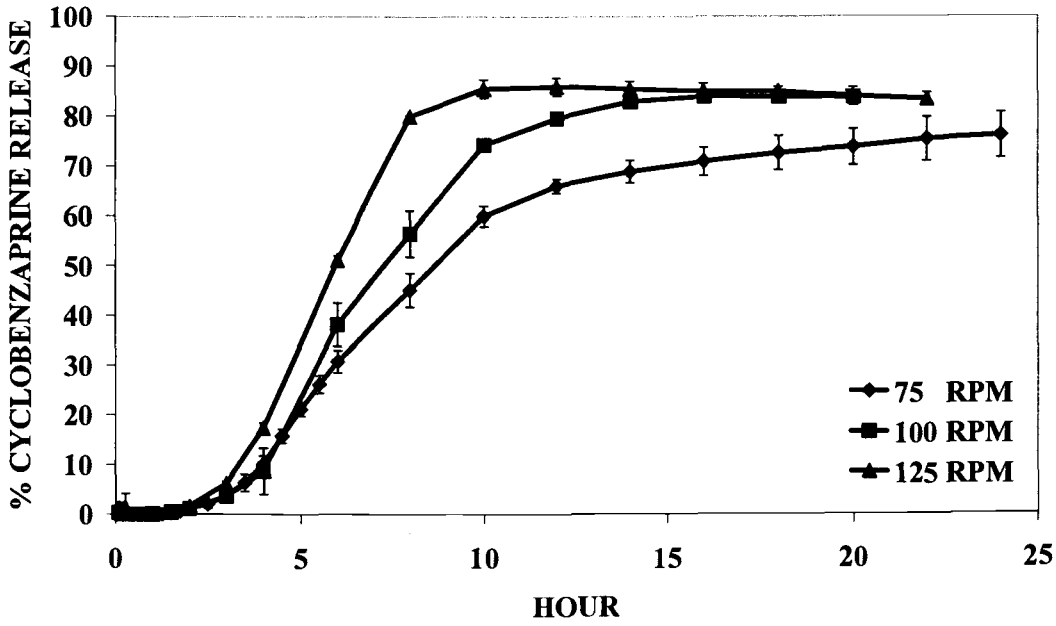


**Figure 5.12: Effect of paddle speed on release of cyclobenzaprine HCL from matrix tablets containing 12% HPMC-E5 and 12% coating. Mean ± SD (n=3).**

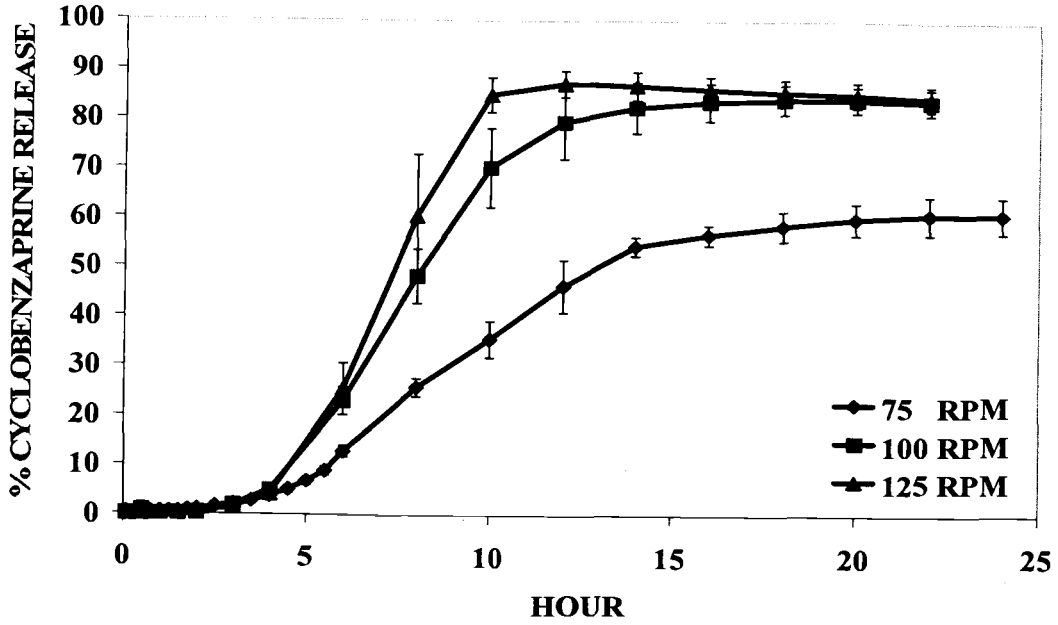


**Figure 5.13: Effect of paddle speed on release of cyclobenzaprine HCL from matrix tablets containing 12% HPMC-E5 and 18% coating. Mean ± SD (n=3).**

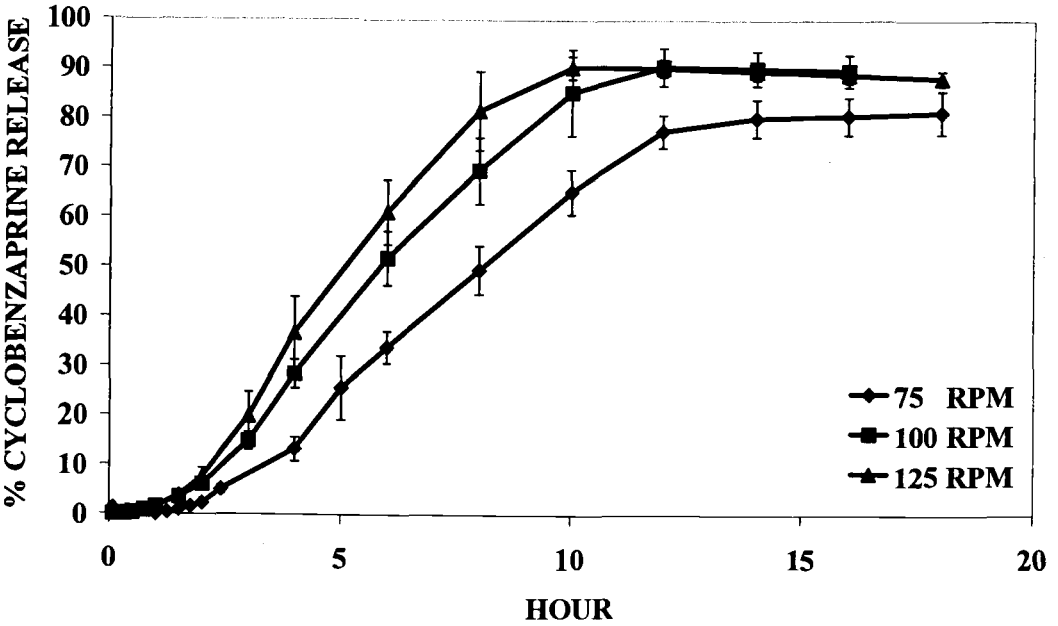


**Figure 5.14: Effect of paddle speed on release of cyclobenzaprine HCL from matrix tablets containing 24% HPMC-E5 and 6% coating. Mean ± SD (n=3).**

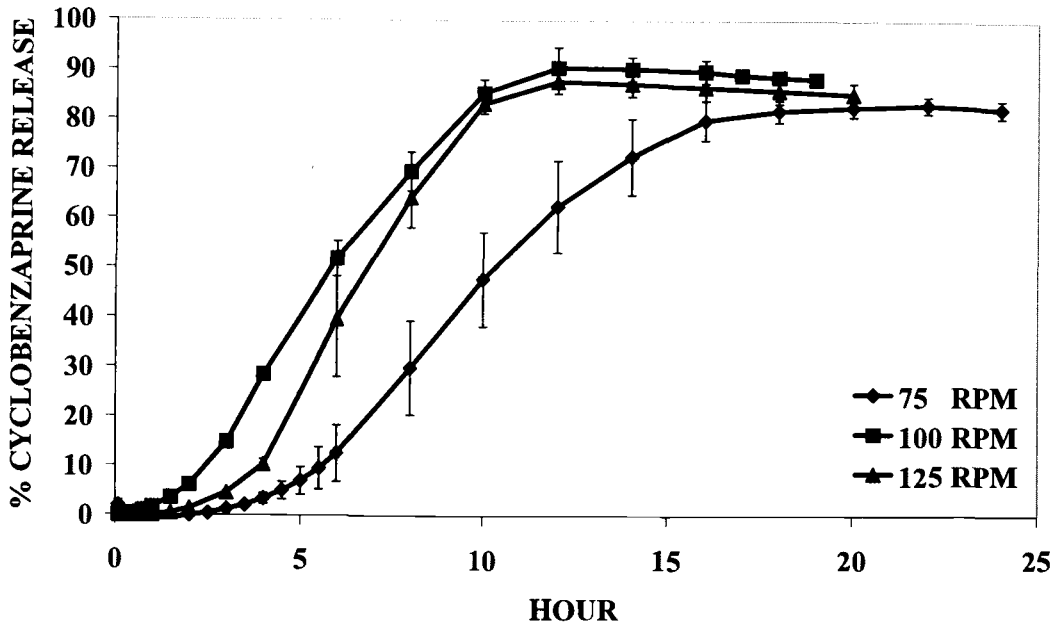


**Figure 5.15: Effect of paddle speed on release of cyclobenzaprine HCL from matrix tablets containing 24% HPMC-E5 and 12% coating. Mean ± SD (n=3).**

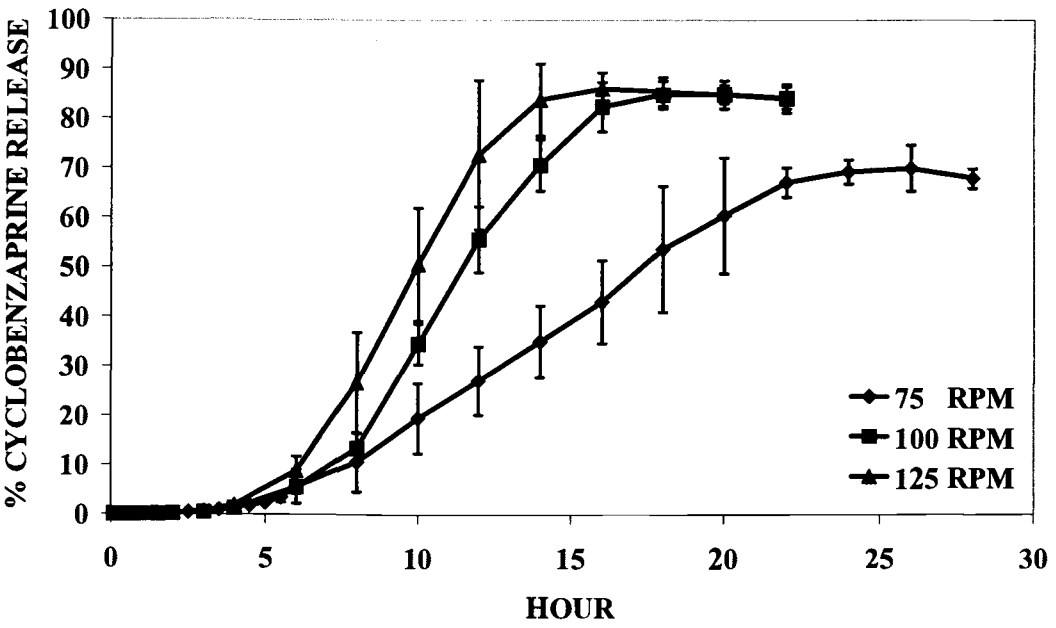


**Figure 5.16: Effect of paddle speed on release of cyclobenzaprine HCL from matrix tablets containing 24% HPMC-E5 and 18% coating. Mean ± SD (n=3).**

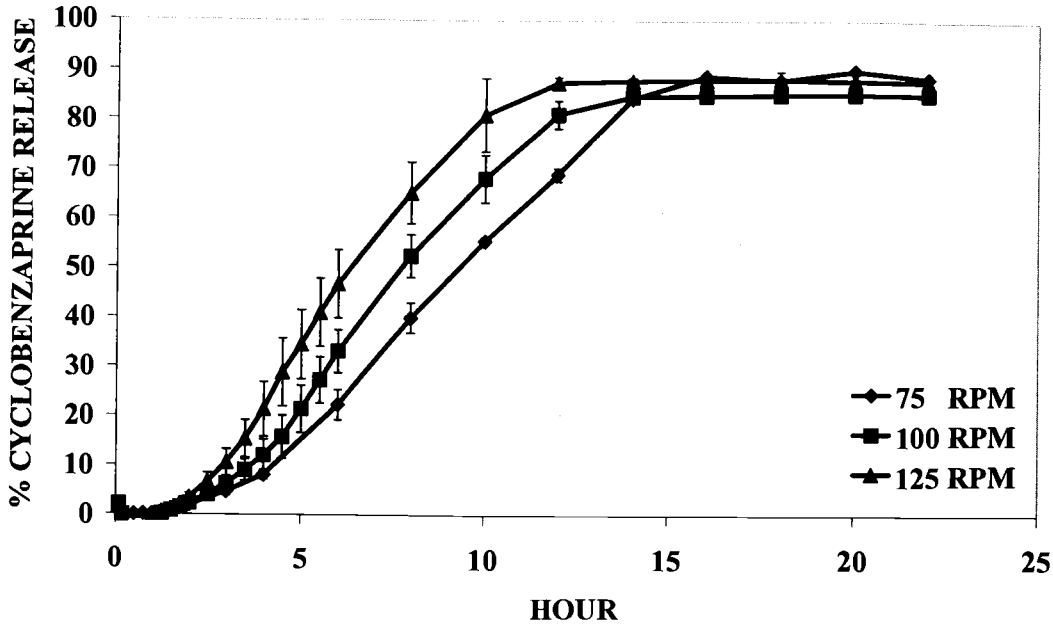


**Figure 5.17: Effect of paddle speed on release of cyclobenzaprine HCL from matrix tablets containing 30% HPMC-E5 and 6% coating. Mean ± SD (n=3).**

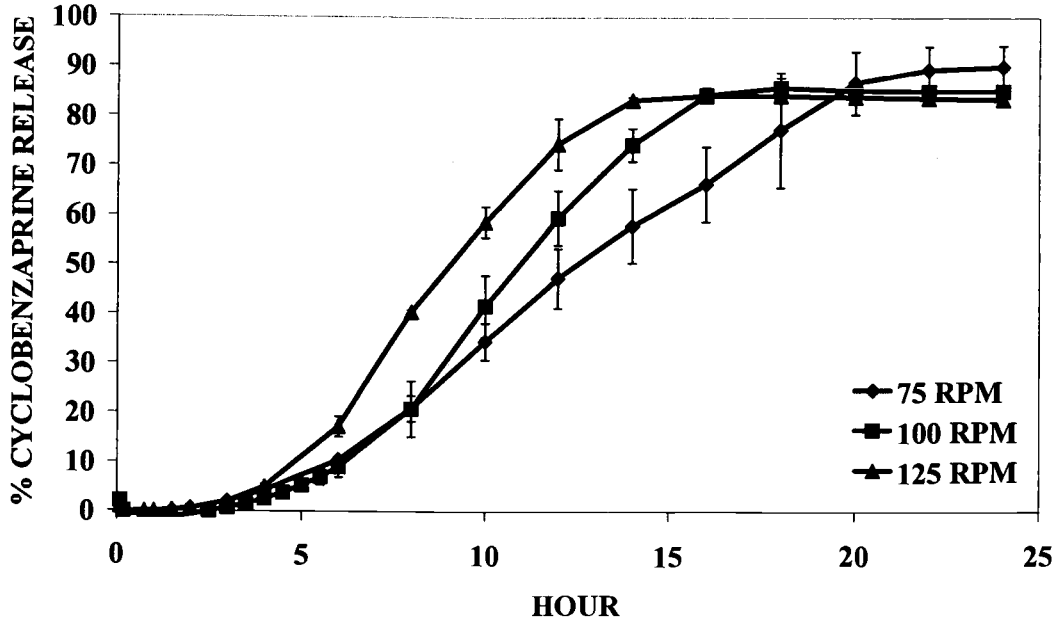


**Figure 5.18: Effect of paddle speed on release of cyclobenzaprine HCL from matrix tablets containing 30% HPMC-E5 and 12% coating. Mean ± SD (n=3).**

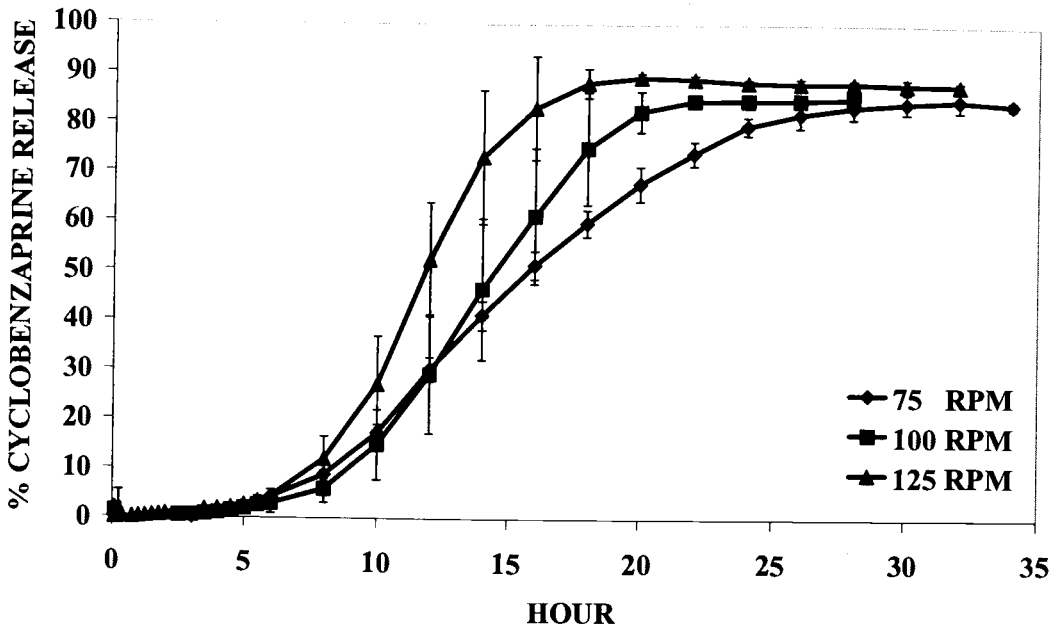


**Figure 5.19: Effect of paddle speed on release of cyclobenzaprine HCL from matrix tablets containing 30% HPMC-E5 and 18% coating. Mean ± SD (n=3).**

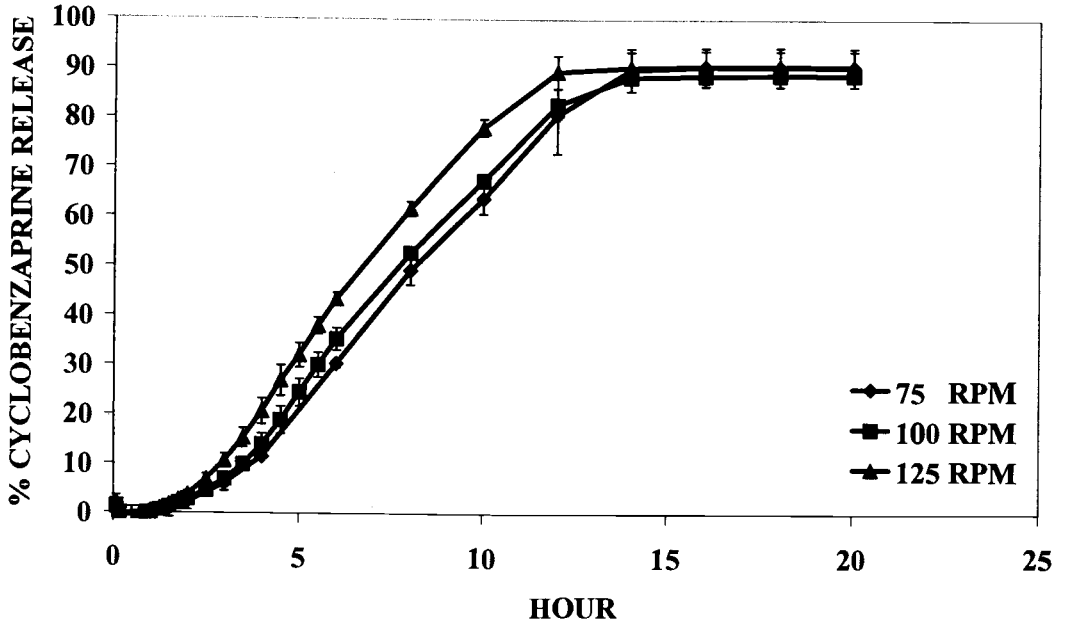


**Figure 5.20: Effect of paddle speed on release of cyclobenzaprine HCL from matrix tablets containing 36% HPMC-E5 and 6% coating. Mean ± SD (n=3).**

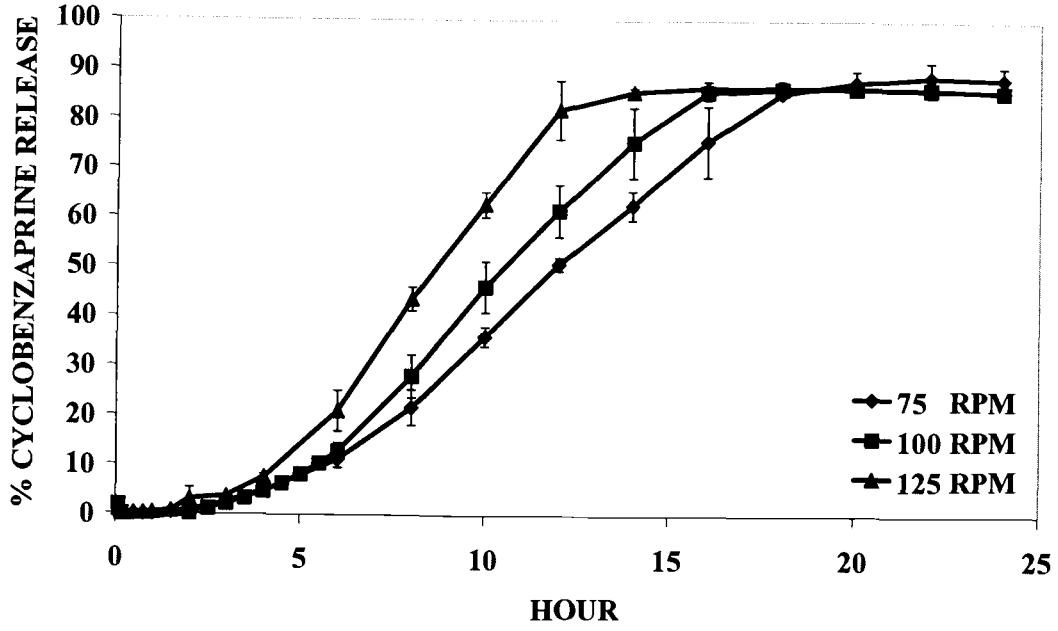


**Figure 5.21: Effect of paddle speed on release of cyclobenzaprine HCL from matrix tablets containing 36% HPMC-E5 and 12% coating. Mean ± SD (n=3).**

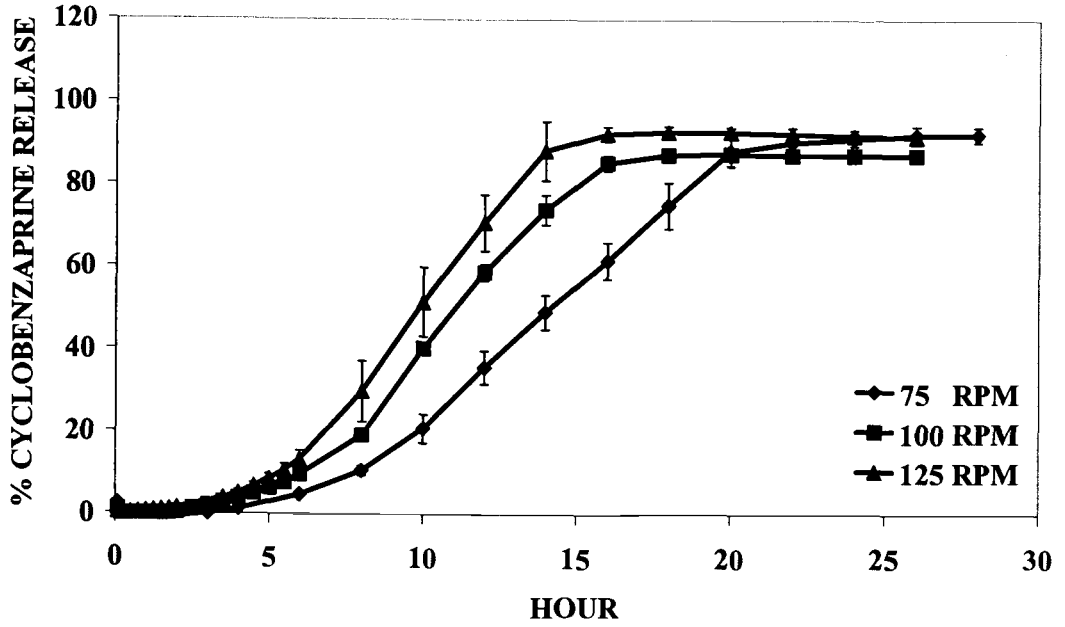


**Figure 5.22: Effect of paddle speed on release of cyclobenzaprine HCL from matrix tablets containing 36% HPMC-E5 and 18% coating. Mean ± SD (n=3).**

## II. Statistical Analysis (Ramsey and Schafer, 1997)

### Determination of Input Variables for Statistical Analysis

The calculated n-value, lag time, % drug release at 6 hours, and % drug release at 10 hours obtained from *in vitro* dissolution profile at 75 rpm of each matrix tablet formulation is shown in Table 5.5-5.8.

**Table 5.5: Calculated n-value of MT1, MT3, and MT4 at 0%, 3%, 6%, 9%, 12%, 15%, and 18% coating**

| % HPMC in core tablet | % Coating | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0% | 3% | 6% | 9% | 12% | 15% | 18% |
| 12% | 0.4138 | 1.0267 | 0.917 | 1.424 | 1.1901 | 1.7835 | 1.1644 |
| | 0.4271 | 1.0292 | 0.9574 | 1.3693 | 1.4844 | 1.3294 | 1.0129 |
| | 0.452 | 1.1351 | 0.775 | 1.4059 | 1.4232 | 1.4608 | 1.1659 |
| 24% | 0.7747 | 1.291 | 1.0575 | 1.3705 | 1.6555 | 1.426 | 1.4007 |
| | 0.798 | 1.2552 | 1.2336 | 1.375 | 1.6348 | 1.7722 | 1.2116 |
| | 0.7705 | 1.2843 | 1.2936 | 1.3198 | 1.5053 | 1.7052 | 1.4001 |
| 36% | 0.8872 | 1.7346 | 1.2661 | 1.6201 | 1.3646 | 1.6513 | 1.5721 |
| | 0.8552 | 1.3977 | 1.4382 | 1.6728 | 1.5978 | 1.4579 | 1.6197 |
| | 0.8463 | 1.2819 | 1.3509 | 0.9897 | 1.2692 | 1.5187 | 1.5043 |

**Table 5.6: Lag time (hours) for MT1, MT3, and MT4 at 0%, 3%, 6%, 9%, 12%, 15%, and 18% coating**

| % HPMC in Core Tablet | % Coating | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0% | 3% | 6% | 9% | 12% | 15% | 18% |
| | 12% | 0 | 0.25 | 1 | 1.25 | 1.5 | 2 | 3 |
| | | 0 | 0.25 | 1 | 1.25 | 1.5 | 2 | 3 |
| | | 0 | 0.25 | 1 | 1.25 | 1.5 | 2 | 3 |
| | 24% | 0 | 0.45 | 1.5 | 1.75 | 2.5 | 3 | 4 |
| | | 0 | 0.45 | 1.5 | 1.75 | 2.5 | 3 | 4 |
| | | 0 | 0.45 | 1.5 | 1.75 | 2.5 | 3 | 4 |
| | 36% | 0 | 0.5 | 1.5 | 1.75 | 2.5 | 3 | 4 |
| | | 0 | 0.5 | 1.5 | 1.75 | 2.5 | 3 | 4 |
| | | 0 | 0.5 | 1.5 | 1.75 | 2.5 | 3 | 4 |

**Table 5.7: Percent drug release at 6 hours for MT1, MT3, and MT4 at 0%, 3%, 6%, 9%, and 12% coating**

| % HPMC in Core Tablet | % Coating | | | | | |
|---|---|---|---|---|---|---|
| | | 0% | 3% | 6% | 9% | 12% |
| | 12% | 83.40783 | 68.89618 | 52.39564 | 33.17636 | 29.04009 |
| | | 72.53252 | 68.92557 | 54.09895 | 34.85119 | 33.1194 |
| | | 59.62739 | 70.70013 | 43.28631 | 35.85054 | 29.89909 |
| | 24% | 90.36162 | 53.8473 | 37.37983 | 28.30576 | 10.85492 |
| | | 83.49617 | 50.8319 | 30.88554 | 30.22053 | 8.020313 |
| | | 78.7087 | 60.0563 | 33.21011 | 21.76239 | 18.8005 |
| | 36% | 71.1051 | 24.1258 | 30.93184 | 16.62693 | 8.561792 |
| | | 49.80396 | 46.37987 | 29.70254 | 16.79187 | 9.766362 |
| | | 60.45453 | 56.21024 | 30.31719 | 40.79624 | 12.54463 |

**Table 5.8: Percent drug release at 10 hours for MT1, MT3, and MT4 at 6%, 9%, 12%, and 15% coating**

| % HPMC in core tablet | % Coating | | | | | |
|---|---|---|---|---|---|---|
| | | 6% | 9% | 12% | 15% | 18% |
| | 12% | 68.41589 | 72.75045 | 57.47748 | 63.91787 | 33.2347 |
| | | 73.74097 | 73.00024 | 62.16197 | 68.09511 | 32.92367 |
| | | 60.50519 | 71.8853 | 60.26097 | 63.05573 | 40.3143 |
| | 24% | 70.02589 | 71.00658 | 43.72182 | 40.4516 | 19.68803 |
| | | 60.7577 | 70.14842 | 41.12848 | 41.51941 | 20.09506 |
| | | 65.02247 | 63.52828 | 58.40961 | 42.14146 | 12.97712 |
| | 36% | 62.73838 | 49.7072 | 25.53264 | 30.57374 | 22.66648 |
| | | 63.74201 | 53.2274 | 34.49717 | 36.13651 | 16.42922 |
| | | 64.74564 | 74.2687 | 37.70967 | 33.32032 | 23.35619 |

**Regression analysis** (see appendix for detail in statistical analysis)

In this study tablets were prepared at different time periods. Tablet with 0%, 6%, 12%, and 18% coating were prepared first (time 1) and three month later (time 2) tablets with 3%, 9%, and 15% were produced. Drug release from matrix tablets spray-coated with barrier membrane is sensitive to the level of coating as well as the characteristics of barrier membrane itself such as puffiness, porosity, and integrity. Tablets prepared at different time periods may have differences in puffiness, porosity, and integrity of membrane barrier according to operating technique during coating process which can affect drug release profile, consequently greater variation in the data. It is noted that time effect in the regression model is not attributed to the effect of month difference. Since the purpose of this study is to create an inferential model that can be used in prediction an *in vitro* drug release from matrix tablets, the important issue involves the inferences that can be drawn to a wider population of tablet prepared at any time period. Thus, we would prefer consider time effect as a random effect rather than fixed effect. However, in this study matrix tablets were prepared on only two different time periods, which is not justified to assume that time, have an effect randomly selected from a population of time periods. Thus, the regression models with a fixed effect of time were examined. Mathematical model equations for predicting n-value, lag time, and percent drug release at 6 and 10 hours are shown in equation 7-10.

N-value = $0.56125 + 0.01101\ hpmc + 0.09333\ coat - 0.00330\ coat^2$ equation (7)

Lag time = $-1.14225 + 0.1018\ hpmc + 0.1353\ coat + 0.002480\ (hpmc \times coat) - 0.0021\ hpmc^2$ equation (8)

There is no significant difference in DR 6 hrs among tablets prepared on different time periods (p-value <0.8406, see appendix). Thus model equation for DR 6 hrs was generated without time effect.

DR6hr = $90.0855 - 0.7380\ hpmc - 6.3939\ coat + 0.1565\ coat^2$ equation (9)

DR10hr = $91.77165 - 0.7586\ hpmc - 0.1399\ coat^2$ equation (10)

**Validation of mathematical models**

When 50% of drug was released within 8 hours, the DR 6 hrs equation (equation 9) was used in predicting drug release profile. Otherwise, the DR 10 hrs equation (equation 10) was used. A new dosage form with 30% HPMC at 0%, 3%, 6%, 9%, 12%, 15%, and 18% coating amount was made and tested. Tablets made with 30% HPMC at 0%, 3%, 6%, and 9% coating demonstrated a similar correlation of observed versus predicted release profiles (Figure 5.23-5.26). The difference between predicted and observed value of lag time and percent drug release was less

than 15 minutes (Table 5.9) and 20%, respectively in all tablet formulations tested except for tablet coated with 12% (>20%). Based on the FDA guidance for bioequivalence study, two dosage formulations that have difference in maximum concentration (Cmax) of equal or less than 20% will not have a clinically significant difference. Lack of fit was observed with dosage form prepared with 30% HPMC at 12%, 15%, and 18% coating (Figure 5.27-5.29). The deviation of predicted release profile from the observed value was more sensitive at higher amount of coating but predicted drug release was still excellent (see Figure 5.29).

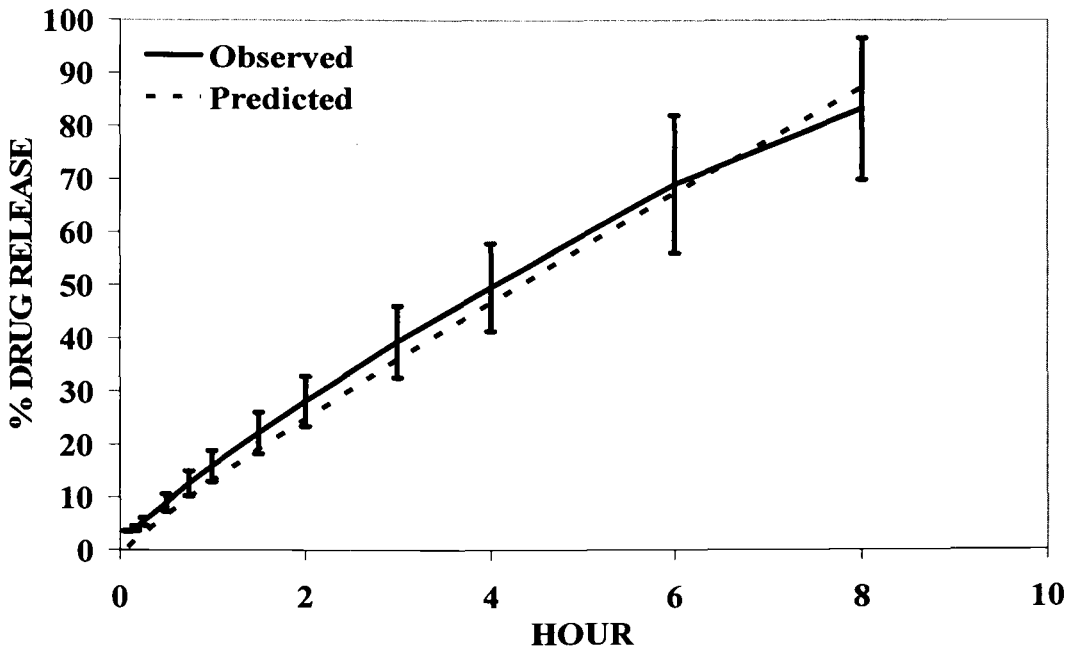


**Figure 5.23: Release of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 0% coating. Mean ± SD (n=3).**

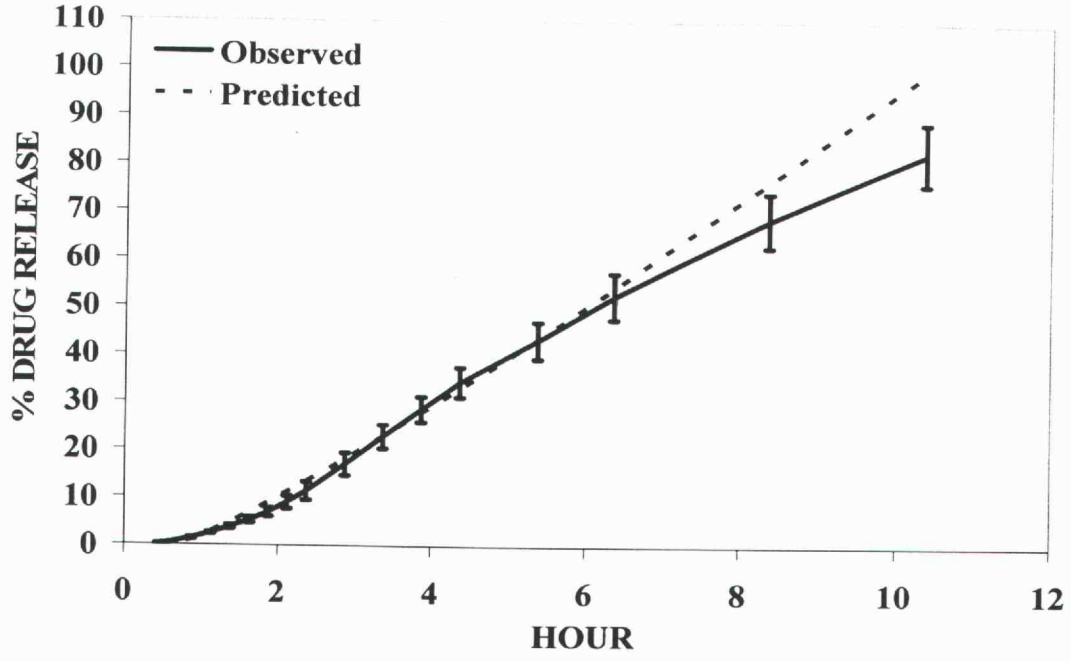


**Figure 5.24: Release of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 3% coating. Mean ± SD (n=3).**

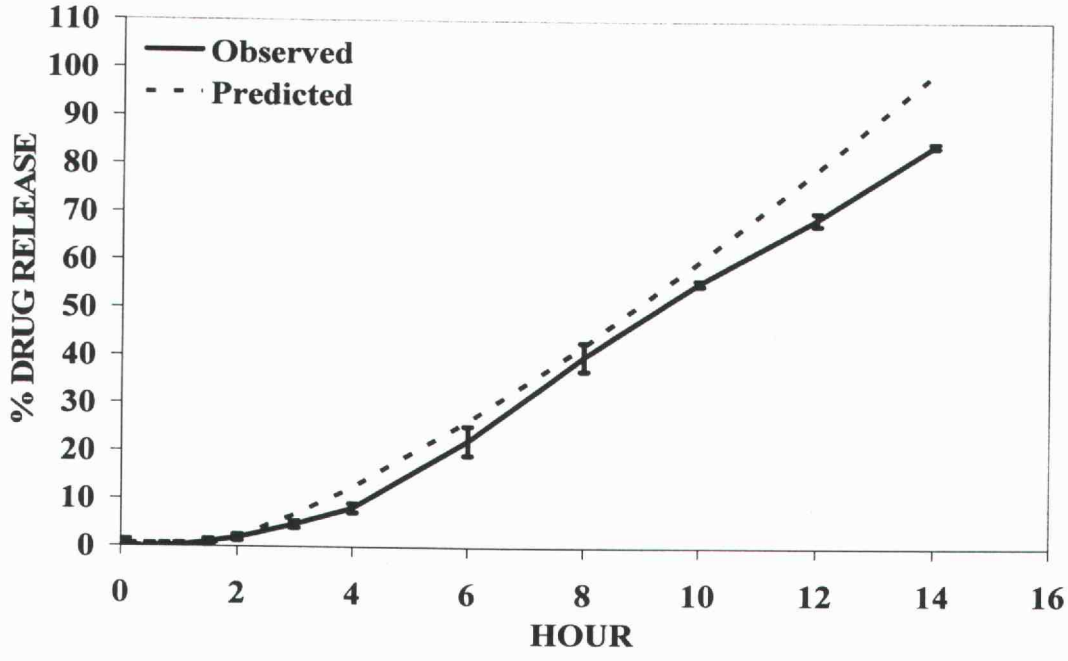


**Figure 5.25: Release of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 6% coating. Mean ± SD (n=3).**

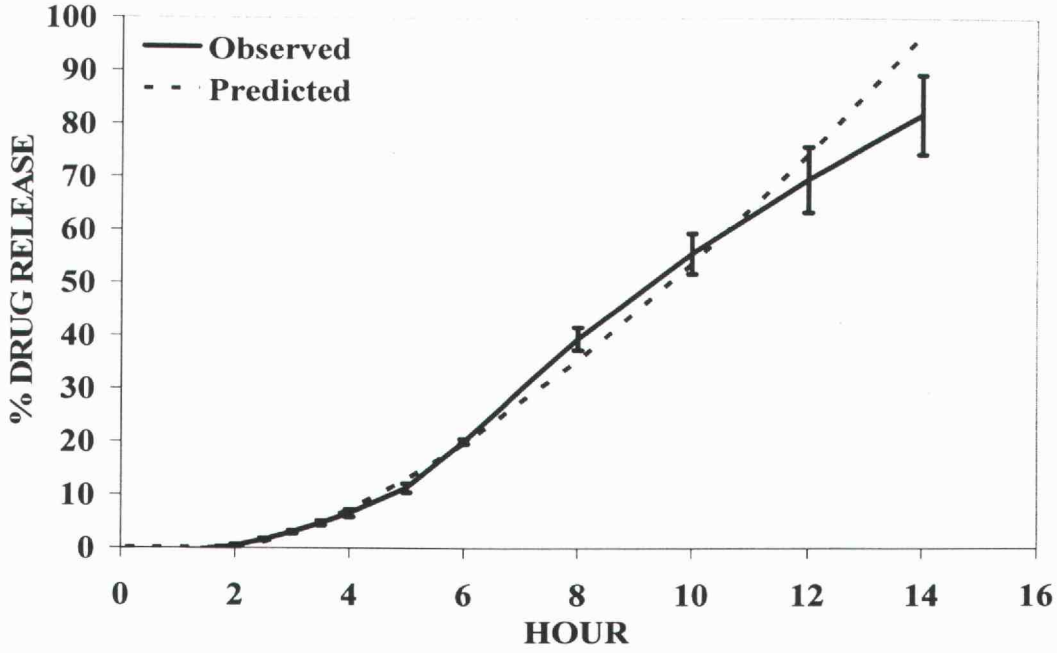


**Figure 5.26: Release of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 9% coating. Mean ± SD (n=3).**

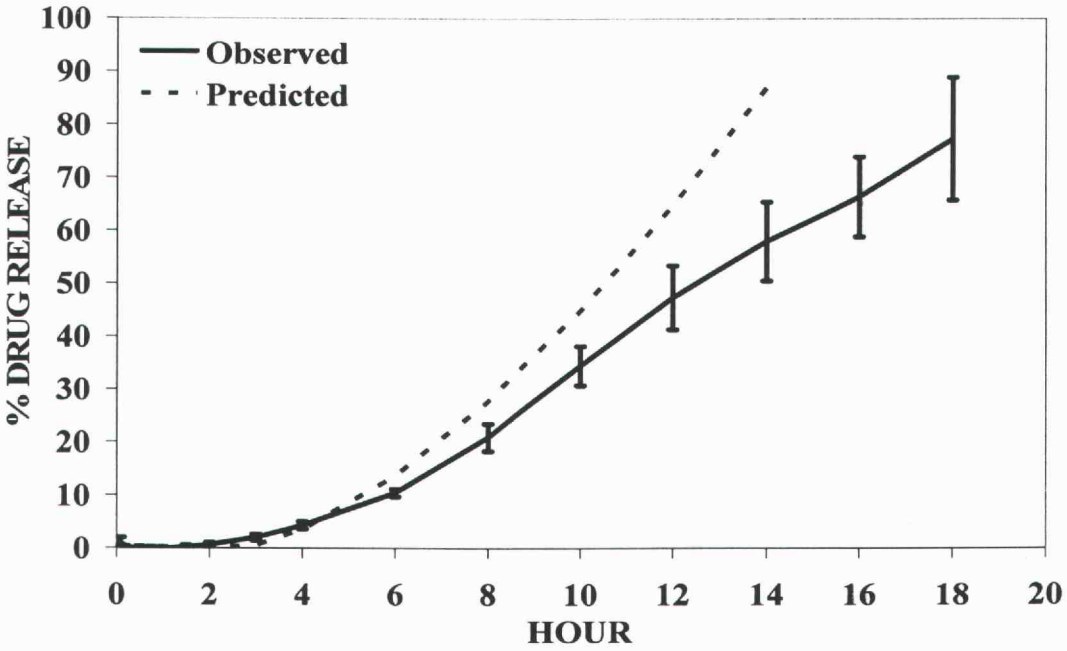


**Figure 5.27: Release of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 12% coating. Mean ± SD (n=3).**

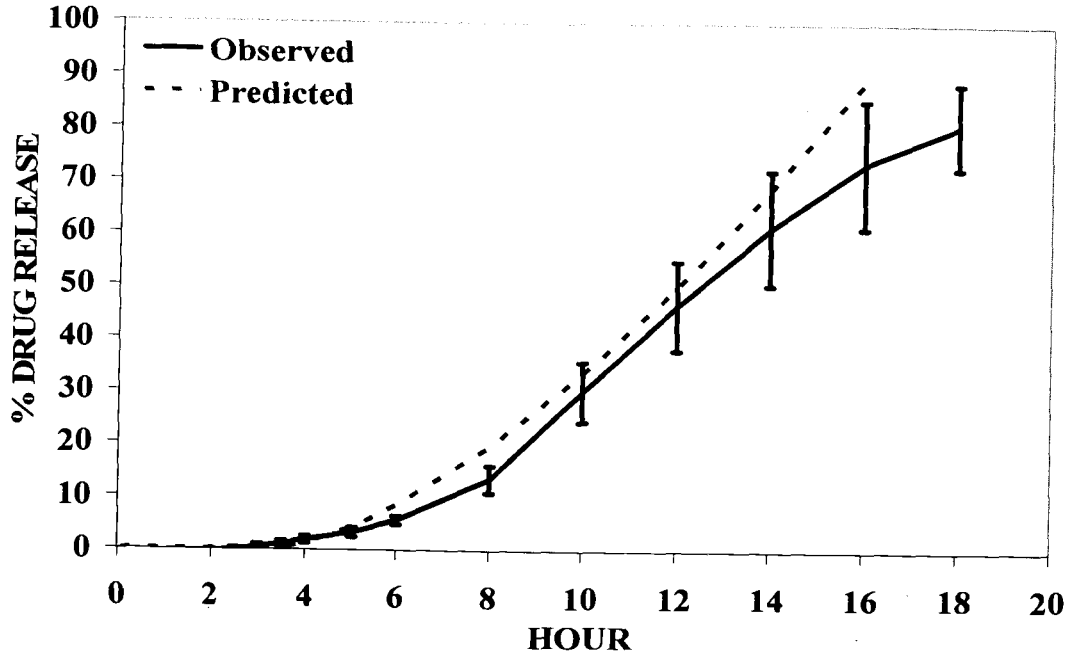


**Figure 5.28: Release of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 15% coating. Mean ± SD (n=3).**

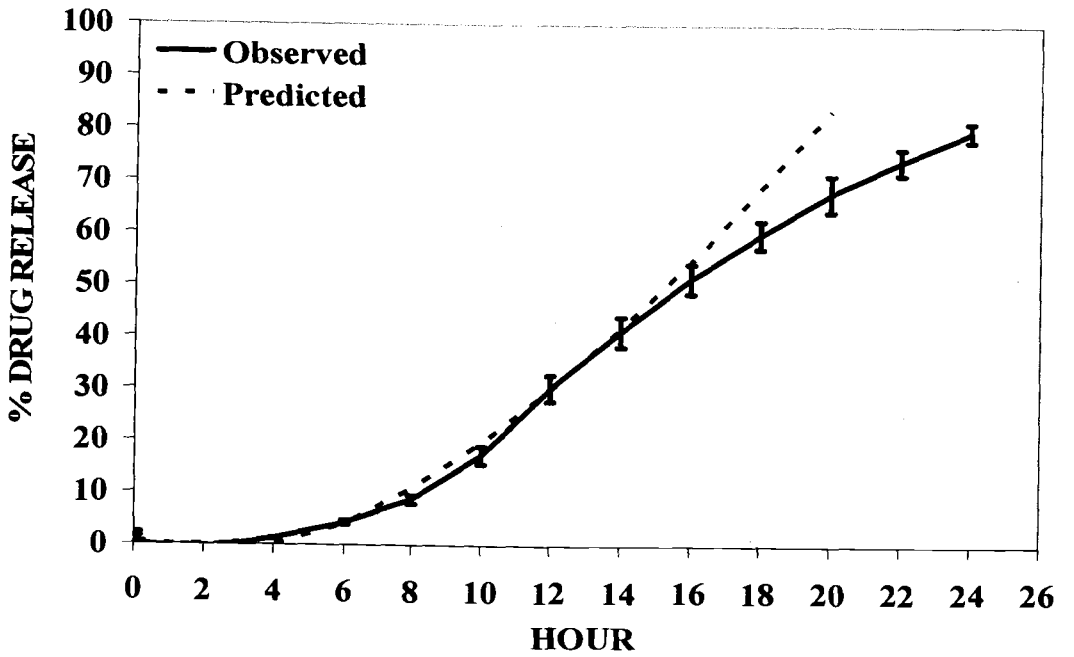


**Figure 5.29: Release of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 18% coating. Mean ± SD (n=3).**

**Table 5.9: Comparison of predicted and observed value**

| Coating Amount | Parameter | Observed (O) | Predicted (P) | Difference (O-P) |
|---|---|---|---|---|
| 0% | N-value | 0.81 | 0.89 | 0.08 |
| | Lag time (minutes) | 0.00 | 3.47 | 3.47 |
| | DR 6 hrs | 69.02 | 67.68 | 1.34 |
| 3% | N-value | 1.20 | 1.14 | 0.05 |
| | Lag time (minutes) | 27 | 41.21 | 14.21 |
| | DR 6 hrs | 48.26 | 51.25 | 2.99 |
| 6% | N-value | 1.49 | 1.33 | 0.16 |
| | Lag time (minutes) | 90 | 78.96 | 11.04 |
| | DR 10 hrs | 55.15 | 59.98 | 4.83 |
| 9% | N-value | 1.49 | 1.46 | 0.03 |
| | Lag time (minutes) | 105 | 116.7 | 11.7 |
| | DR 10 hrs | 55.60 | 53.68 | 1.92 |

**Table 5.9: Comparison of predicted and observed value (continued)**

| Coating Amount | Parameter | Observed (O) | Predicted (P) | Difference (O-P) |
|---|---|---|---|---|
| 12% | N-value | 1.35 | 1.54 | 0.19 |
| | Lag time (minutes) | 150 | 154.45 | 4.45 |
| | DR 10 hrs | 34.45 | 44.87 | 10.42 |
| 15% | N-value | 1.68 | 1.55 | 0.13 |
| | Lag time (minutes) | 180 | 192.2 | 12.2 |
| | DR 10 hrs | 30.09 | 33.54 | 3.44 |
| 18% | N-value | 1.34 | 1.5 | 0.16 |
| | Lag time (minutes) | 240 | 229.94 | 10.06 |
| | DR 10 hrs | 17.30 | 19.69 | 2.38 |

# CONCLUSION

Results showed that drug release characteristics, release rate and lag time, were influenced and could be controlled by modifying the amount of hydrophilic materials such as HPMC and Pectin in the core tablet and the barrier thickness. Zero-order release of cyclobenzaprine hydrochloride with a desired lag time could be achieved by varying the parameters mentioned above. Sustained/controlled release of cyclobenzaprine hydrochloride can be tailored to produce a desired release rate using hydrophilic matrix tablets coated with a barrier membrane that forms a support platform in situ. Study of paddle speed effect on release rate of cyclobenzaprine hydrochloride indicated that drug release rate was often increased as paddle speed increased, but that a formulation relatively insensitive to paddle speed could also be produced. Statistical analysis shows that drug release characteristics (n-value, lag time, % drug release at 6 hours and 10 hours) are a function of time periods. Tablets prepared at difference time periods may have differences in puffiness, porosity, and integrity of membrane barrier according to operating technique during coating process which can affect drug release profile, consequently greater variation in the data. This variation can be minimized by controlling the operating factors in the coating process. Mathematical equations generated from regression analysis of n-value, lag time, and % drug release as a function of hydrophilic material amount and coating amount after accounting for the effect of time can be used as a tool for predicting and optimizing *in*

*vitro* drug release of cyclobenzaprine hydrochloride from matrix tablets spray-coated with a barrier membrane in product development.

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## Chapter 6. Conclusion

A novel formulation for oral DNA vaccine delivery was developed for Rainbow trout by combining the usefulness of non-viral vectors (polycationic liposomes and polycationic polymer) to facilitate cellular membrane uptake of the DNA and the embedding the DNA into enteric-coated microparticles to prevent DNA degradation in the gastrointestinal tract environment. Spray drying and spray-coated beads techniques were employed in the enteric-coated microparticles preparation. The spray drying technique allowed production of spherical shape enteric-coated microparticles with a particle size range from 0.18 to 20 μm. Larger particle sizes of 40-50 mesh were obtained from the spray-coated beads technique. Eudragit® L-30D, HPMCP-55, and CAP all proved to be good candidates for the preparation of the enteric-coated microparticulate oral vaccine. Eudragit® L-30 D was preferred due to it enteric-coated properties along with it being less sticky. The amount of enteric-coated polymer needed to ensure the enteric protection of the DNA vaccine was found to be 15 percent, and 20 percent for spray-dried microparticles and spray-coated beads delivery systems, respectively.

Transfection of DNA in liposomes composed of DOTAP/DOPE and cationic polymer (PEI) were found to provide a high level of gene expression in both caco-2 cells and CHSE-214 cells in our study. Cells transfected with DNA complexed with PEI and with DOTAP/DOPE demonstrated 2-600 fold higher expression level in the cell lines compared to cells transfected with the complexes of DNA and a commercial transfection reagent (lipofectin®). Complexation of DNA with DOTAP/DOPE and

with PEI also prevented DNA degradation from DNase. As confirmed by agarose gel electrophoresis, DNA complexed with DOTAP/DOPE or with PEI were not degraded after exposing them to DNase 1 for at least 30 minutes, while naked DNA was quickly degraded in 2 minutes.

Plasmid DNA extracted from the enteric-coated microparticles retained their structural and functional integrity as confirmed by gel electrophoresis and transfection assay in CHSE-214 cells. Both relaxed and supercoiled forms of plasmid IHNV were observed. Cells transfected with extracted plasmid DNA showed gene expression level in the cell lines of 62.63% compared to gene expression level of 100% from control plasmid DNA

*In vivo* experiment showed a high mortality (> 80%) in fish vaccinated orally with spray-dried enteric protected microparticles (particle size range of 0.18-20 μm). Fish immunized with spray-coated enteric protected beads (particles size range of 290-420 μm) demonstrated less mortality (55%). A statistically significant difference in mortality between oral treatment versus oral blank treatment (p-value < 0.002, chi-test) was observed suggesting an improvement in the level of protection against IHNV when fish were vaccinated with spray-coated enteric protected beads. The efficacy of protection in fish immunized by spray-coated enteric protected beads however, can not be directly compared to the efficacy of fish immunized by spray-dried enteric protected microparticles because the experiments were performed separately with different virus strains and virus concentrations. Further experiments are needed to compare the efficacy of spray-dried enteric protected vaccine microparticles and the

spray-coated enteric protected vaccine beads delivery systems in inducing an immune response against IHNV. The efficacy of both spray-dried enteric protected microparticles and spray-coated enteric protected beads vaccine systems in inducing an immune response was not as effective as intramuscular vaccine injection. However, an improvement in a survival rate with the use of spray-coated enteric protected beads suggests that a successful vaccine delivery system can be developed using larger particle size microparticles. In addition, more research in finding effective vector carriers for DNA vaccine to facilitate membrane uptake of the DNA and in delivery systems that can prevent the denaturation of plasmid DNA is needed in order to develop a safe, effective, and commercially viable vaccine to control future outbreaks of IHNV.

A novel controlled release delivery system based on hydrophilic matrix tablets having a membrane coating have been previously developed in our laboratory. The release mechanism of this delivery system depends upon the swelling of matrix tablet in the gastrointestinal tract as well as the rupture of membrane coating in the belly-band area of the tablets and the retention of platform supports on lateral surfaces of the tablets. It was found that drug release characteristics, release rate and lag time, was influenced and can be controlled by modifying the amount of hydrophilic materials such as HPMC and Pectin in the core tablet and the coating barrier thickness surrounding the tablet. Zero-order drug release with a desired lag time can be achieved by varying the parameters mentioned above. Mathematical equations generated from regression analysis of n-value, lag time, and percent drug release as a

function of the amount of hydrophilic material and coating amount after accounting for the time period effect could be used in product development as a model for predicting and optimizing *in vitro* drug release of cyclobenzaprine hydrochloride from matrix tablets spray-coated with a barrier membrane. The newly developed equations are:

$$\text{N-value} = 0.56125 + 0.01101\ \text{hpmc} + 0.09333\ \text{coat} - 0.00330\ \text{coat}^2$$

$$\text{Lag time} = -1.14225 + 0.1018\ \text{hpmc} + 0.1353\ \text{coat} + 0.002480\ (\text{hpmc} \times \text{coat}) - 0.0021\ \text{hpmc}^2$$

$$\text{DR6hr} = 90.0855 - 0.7380\ \text{hpmc} - 6.3939\ \text{coat} + 0.1565\ \text{coat}^2$$

$$\text{DR10hr} = 91.7716 - 0.7586\ \text{hpmc} - 0.1399\ \text{coat}^2$$

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# APPENDIX

# Appendix

## Statistical analysis

### *Data exploration for n-value*

Scatterplot of n-value versus % COAT and n-value versus % HPMC is displayed in Figure A.1 and A.2. N-value was found to increase with an increasing in coating level and percent of HPMC in the core tablet. This increasing relationship, however, appears to be slightly nonlinear with the observation of curvature. Coded scatterplot also suggested that the difference in n-value between seven coating levels may be smaller for the higher percent of HPMC than for the lower ones. Consequently, %COAT by %HPMC interaction term was included in the tentative model. Coating amount seems to have more predominant effect on n-value than percent of HPMC in the core tablet. Type III test confirmed that percent HPMC in the core tablet and coating amount has a significant effect on n-value (Table A.1).

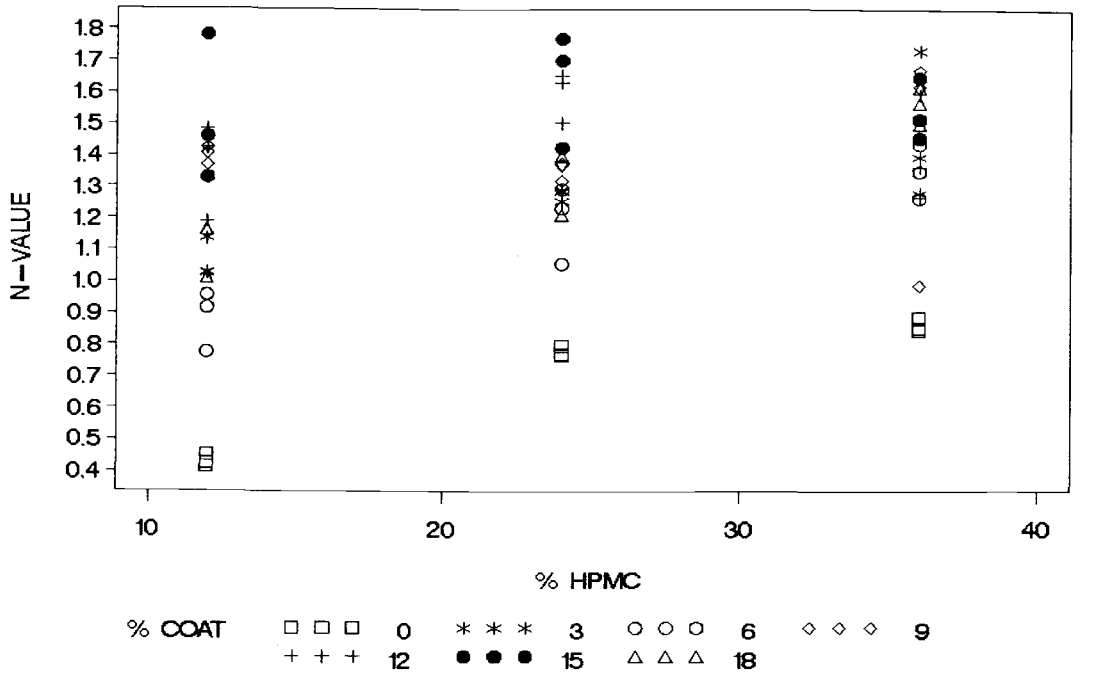


**Figure A.1: Coded scatterplot of n-value versus % HPMC.**

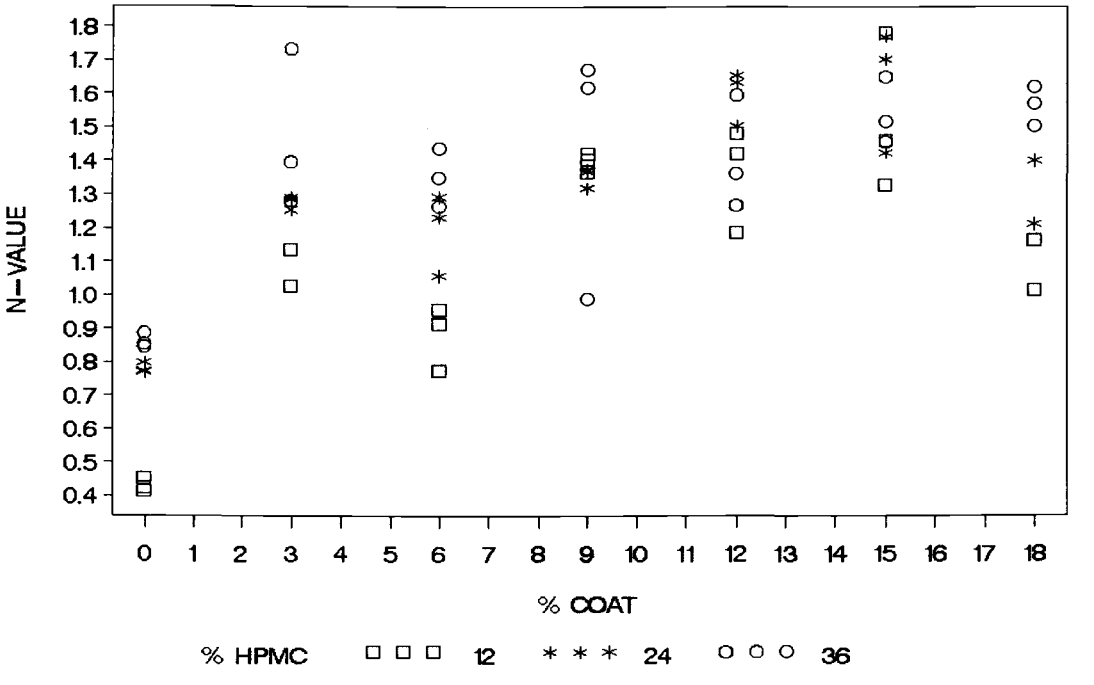


**Figure A.2: Coded scatterplot of n-value versus % COAT.**

**Table A.1: Type III test of n-value with respect to %HPMC and %COAT**

| Source of Variation | DF | Type III SS | Mean Square | F-Value | Pr > F |
|---|---|---|---|---|---|
| %COAT | 6 | 4.45004603 | 0.74167434 | 37.19 | <.0001 |
| %HPMC | 2 | 0.79660048 | 0.39830024 | 19.97 | <.0001 |
| %HPMC * %COAT | 12 | 0.53552825 | 0.04462735 | 2.24 | 0.0271 |

The plot of n-value residual versus % HPMC showed a random distribution of data which suggests no need of transformation or additional of an extra term (Figure A.3). Lack of fit was observed with the residual n-value versus % COAT (Figure A.4). Data transformation and adding of a non-linear term of % COAT (%COAT$^2$) in the regression model did not accommodate the lack of fit (Figure A.5). The pattern of this lack of fit indicates a missing effect, possibly time effect, since each tablet formulation was prepared at different time periods. In this study tablets were prepared at different time periods. Tablet with 0%, 6%, 12%, and 18% coating were prepared first (time 1) and three months later (time 2) tablets with 3%, 9%, and 15% were produced. Drug release from matrix tablets spray-coated with barrier membrane is sensitive to the level of coating as well as the characteristics of barrier membrane itself such as puffiness, porosity, and integrity. Tablets prepared at different time periods may have differences in puffiness, porosity, and integrity of membrane barrier according to operating technique during coating process which can affect drug release profile, consequently greater variation in the data. It is noted that time effect in the regression model is not the effect of month difference.

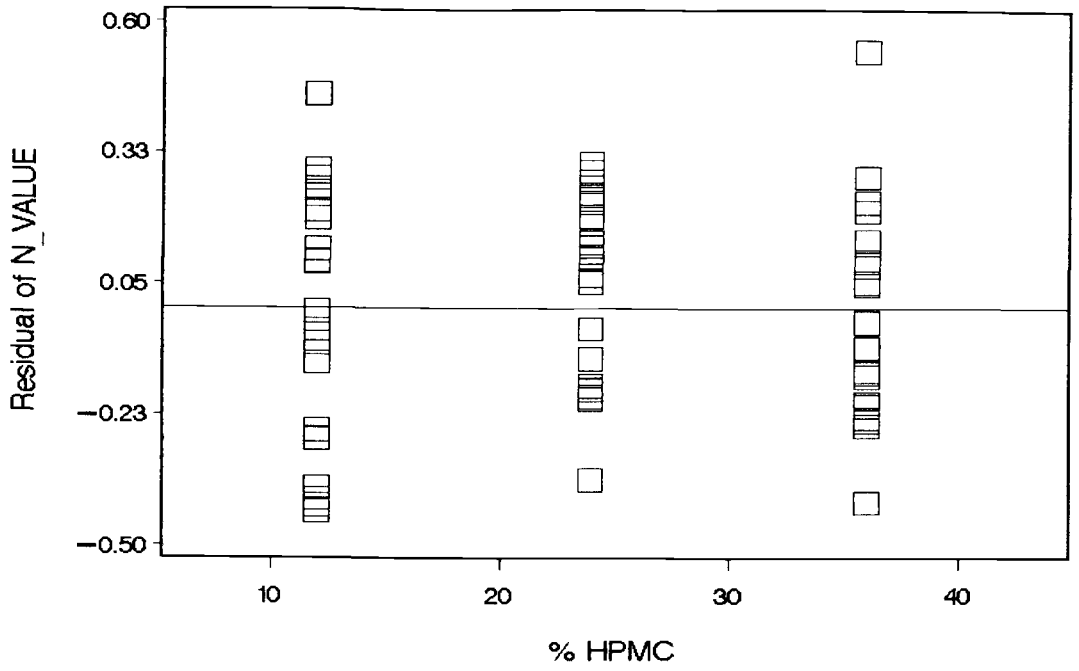


**Figure A.3: The plot of n-value residual with respect to % HPMC (random distribution).**

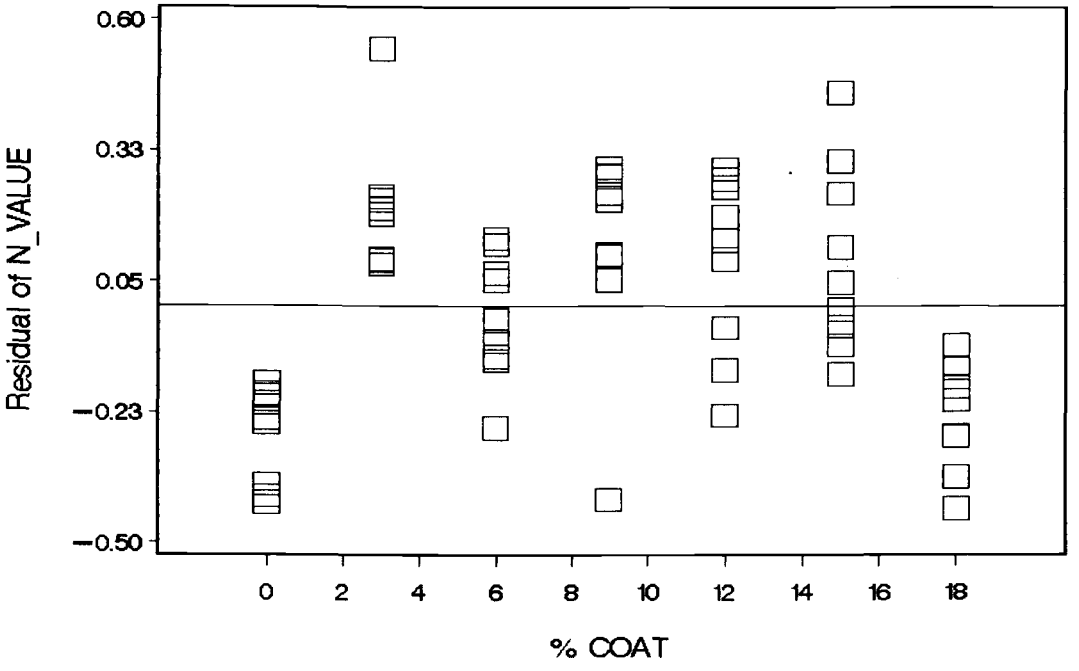


**Figure A.4: The plot of n-value residual with respect to % COAT (non-random distribution).**

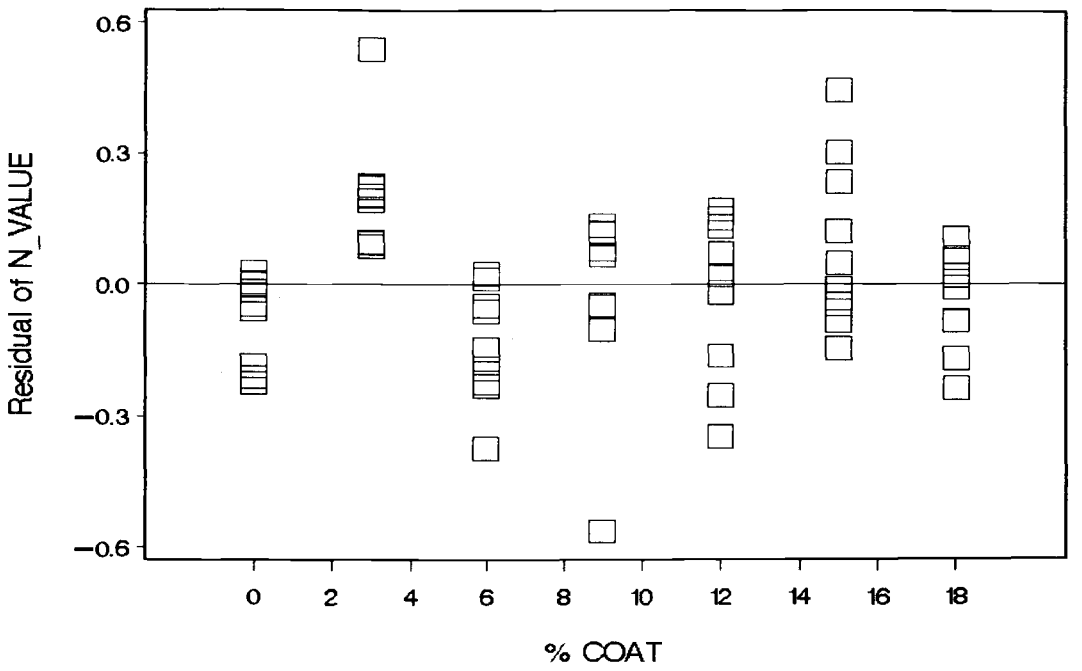


**Figure A.5: The plot of n-value residual with respect to % COAT (with the extra term of %COAT$^2$).**

**Regression analysis with mixed linear model**

To examine whether lack of fit is caused by the missing of time effect, time was included in the regression model. Analysis of variance table showed that there is a significant difference among tablets prepared at different time periods (p-value <.0001, Table A.2).

**Table A.2: Analysis of variance table for n-value with time effect**

| Source | Sum of Squares | d.f. | Mean Square | F-Statistic | *p*-Value |
|---|---|---|---|---|---|
| % HPMC | 0.73318144 | 1 | 0.73318144 | 18.89 | <.0001 |
| % COAT | 2.61180117 | 1 | 2.61180117 | 67.28 | <.0001 |
| Time | 0.98432447 | 1 | 0.98432447 | 25.36 | <.0001 |
| Residual | 2.29641204 | 59 | 0.03882054 | | |
| Total | 6.61971912 | 62 | | | |

***R*-squared** = 65.4% **Estimated SD** = 0.197029

Since the purpose of this study is to create an inferential model that can be used in prediction an *in vitro* drug release from matrix tablets. The important issue involves the inferences that can be drawn to a wider population of tablets prepared at any time period. Thus, time effects should be considered as random effect rather than fixed effect. However, in this study matrix tablets were prepared on only two different time periods, which does not justified the assumption that time has an effect randomly selected from a population of time periods. Thus, the regression models with a fixed effect of time were examined.

Regression model with a fixed effect of time

The plot of residual n-value versus % COAT after including time effect (Figure A.6) showed that lack of fit resides in a missing non-linear term of % COAT (% $COAT^2$). Additional of %$COAT^2$ in the regression model accommodate this lack of fit (Figure A.7). This result suggests that lack of fit is caused by the missing of

time effect. The plot of n-value residual versus % HPMC indicated a random distribution of data (Figure A.8).

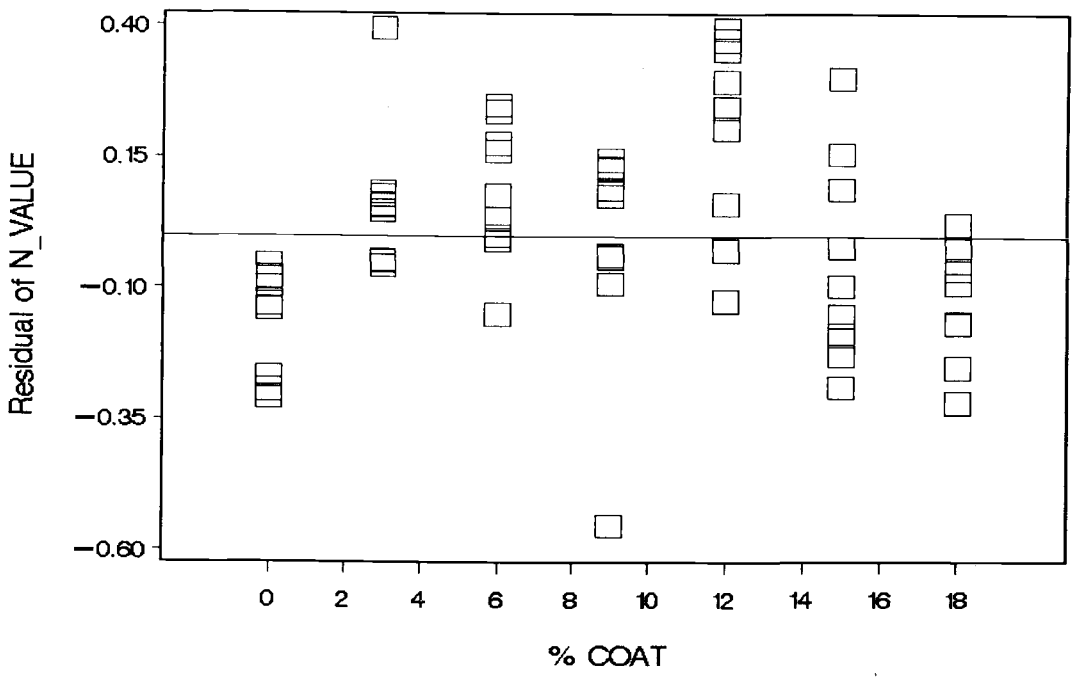


**Figure A.6: The plot of n-value residual with respect to % COAT after account for the fixed effect of time (non-random distribution).**

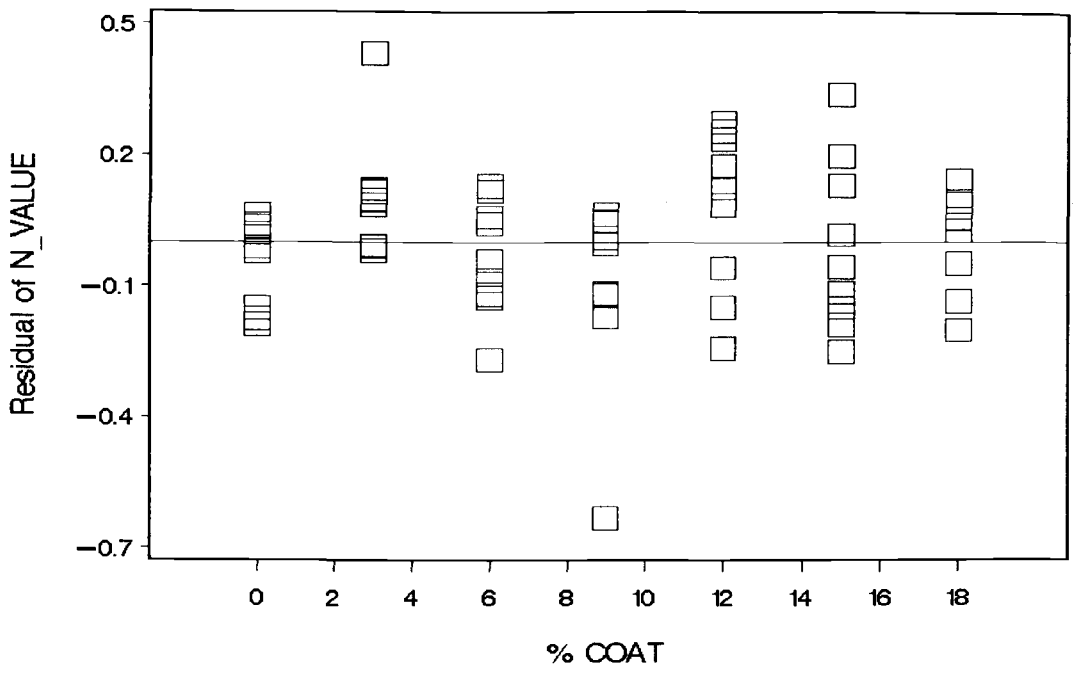


**Figure A.7: The plot of n-value residual with respect to % COAT after account for the fixed effect of time (random distribution with the extra term of $\%COAT^2$).**

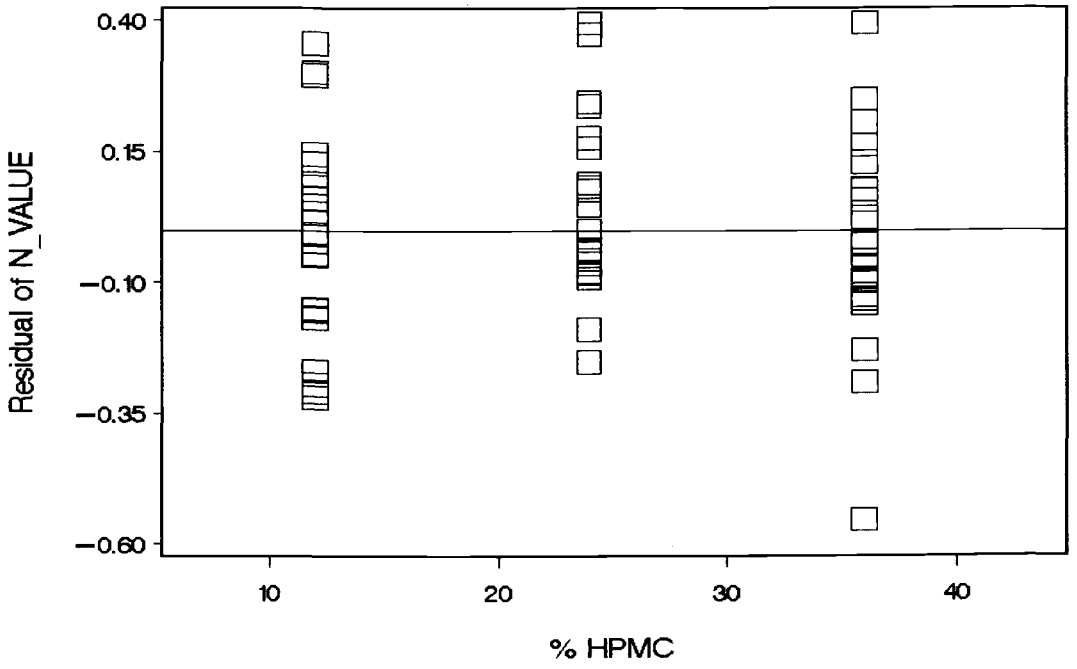


**Figure A.8: The plot of n-value residual with respect to % HPMC after account for the fixed effect of time (non-random distribution).**

To obtain a finer picture about nonconstant variance, the plot of response residual versus its predicted value was created by fitting the tentative regression model. From initial inspection, the tentative regression model for n-value is presented in equation 1-2.

Model without time effect

$$\mu\{\text{N-value} \mid \text{hpmc, coat}\} = \text{hpmc} + \text{coat} + (\text{hpmc} \times \text{coat}) + \text{coat}^2 \qquad \text{equation (1)}$$

Model with time effect

$$\mu\{\text{N-value} \mid \text{hpmc, coat}\} = \text{hpmc} + \text{coat} + (\text{hpmc} \times \text{coat}) + \text{coat}^2 + \text{time} \qquad \text{equation (2)}$$

Where:

hpmc is percent HPMC in the core tablet

coat is the amount of barrier coating.

Residual plot obtained from fitting the tentative model with or without time effect indicated that data has a constant variance (Figure A.9).

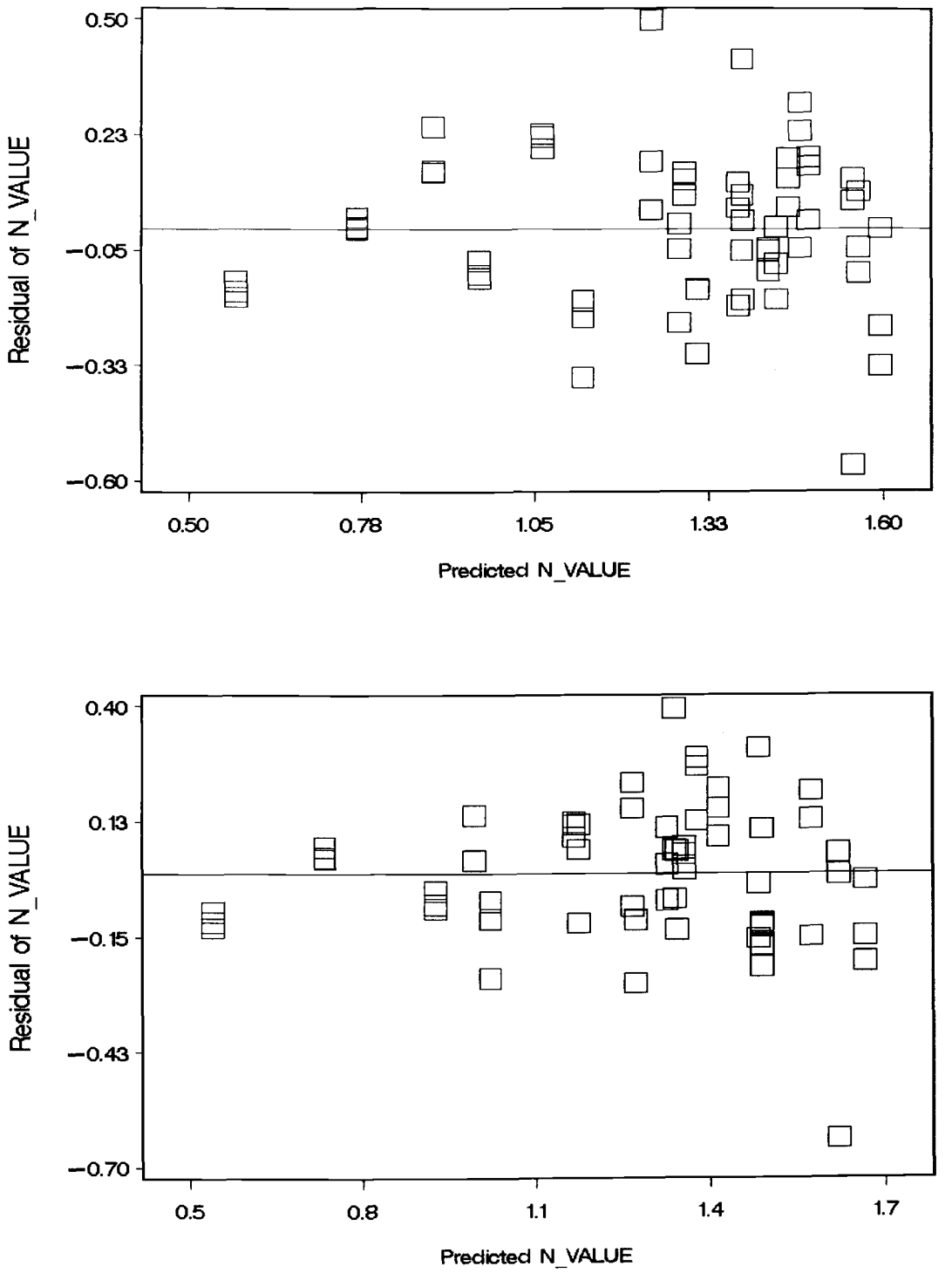


**Figure A.9: The plot of n-value residual versus its predicted value.** Panel A shows the residual plot without time effect; Panel B is the residual plot with the fixed effect of time.

***Data Exploration for Lag Time***

Coded scatterplot of lag time versus % COAT using different codes to represent three levels of % HPMC, 12%, 24%, and 36%, revealed that lag time was increased as coating level increased (Figure A.11). Longer lag times were associated with an increasing amount of HPMC; however, increasing the amount of HPMC to a higher level of 24%-36% was associated with no change in lag times (Figure A.10). Coded scatterplot also suggested that the difference in lag time between three HPMC levels may be greater for the higher amounts of coating than for the lower ones. Consequently, the percent COAT by % HPMC interaction term was included in the tentative model. Type III test showed a significant effect of both % HPMC and % COAT on lag time value (Table A.3).

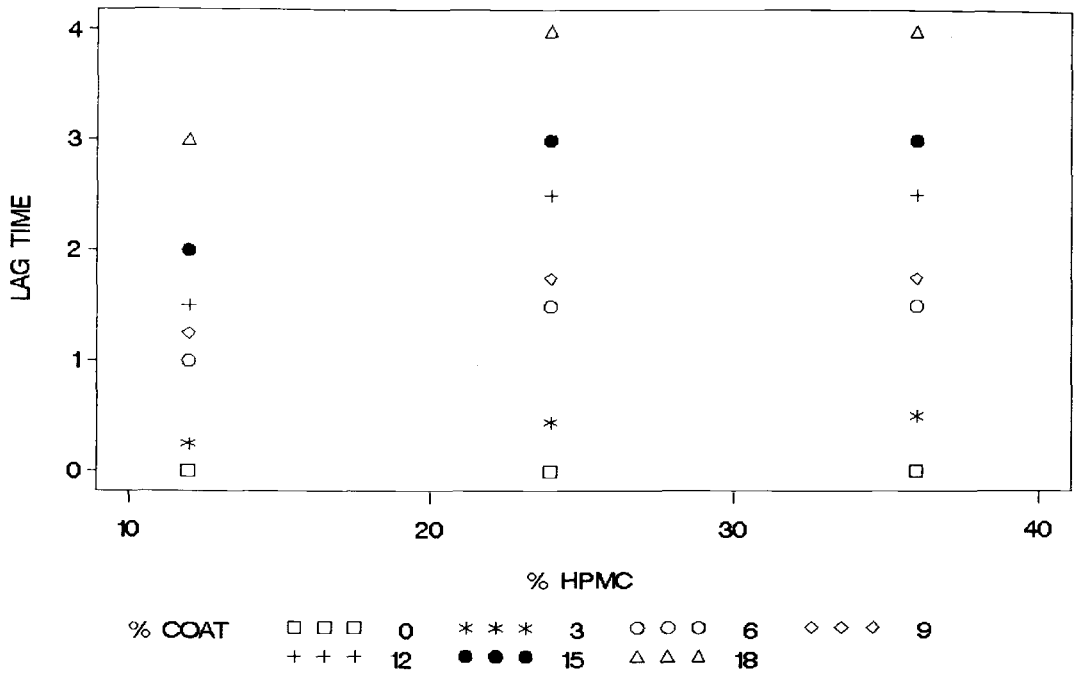


**Figure A.10: Coded scatterplot of lag time versus %HPMC.**

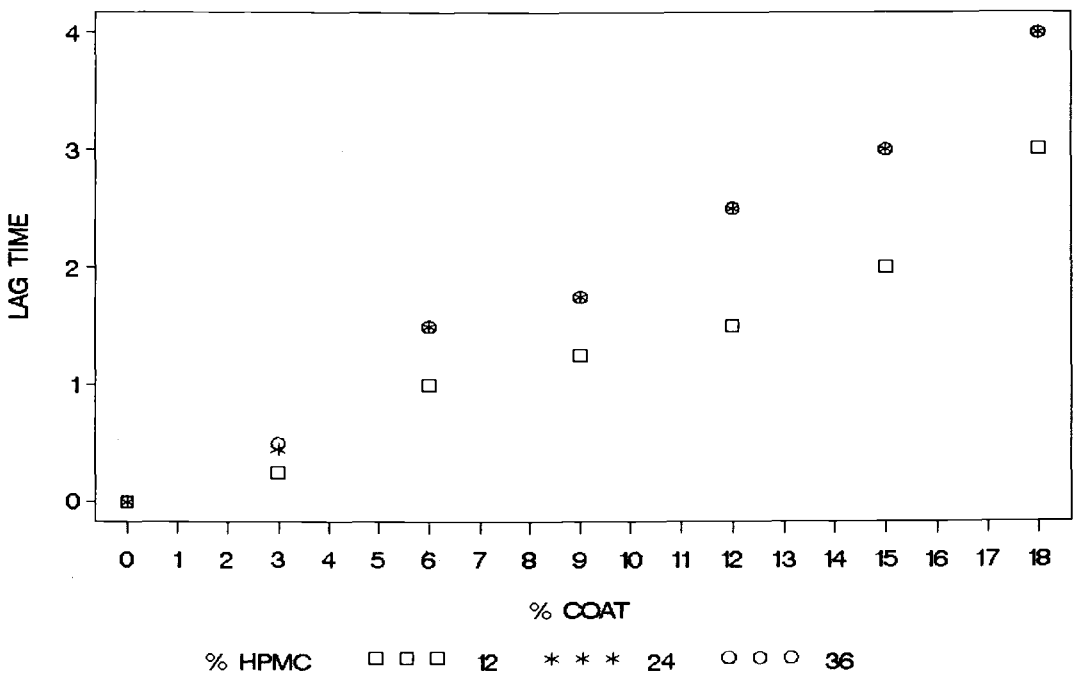


**Figure A.11: Coded scatterplot of lag time versus % COAT.**

**Table A.3: Type III test of lag time with respect to %HPMC and %COAT.**

| Source of Variation | DF | Type III SS | Mean Square | F-Value | Pr > F |
|---|---|---|---|---|---|
| %COAT | 6 | 87.72357413 | 14.62059524 | Infty | <.0001 |
| %HPMC | 2 | 5.10071429 | 2.55035714 | Infty | <.0001 |
| %HPMC * %COAT | 12 | 2.00428571 | 0.16702381 | Infty | <.0001 |

The plot of lag time residual versus % HPMC revealed that lack of fit resides in a missing non-linear term of % HPMC (%HPMC$^2$), Figure A.12. The new regression model with an extra term of %HPMC$^2$ was performed. Residual plot obtained from this new model (Figure A.13) showed that adding %HPMC$^2$ in the regression model accommodates the lack of fit observed in the previous residual plot (residual plot without the %HPMC$^2$ term). The plot of lag time residual versus % COAT indicated a slight lack of fit which may possibly be caused by the missing of time effect (Figure A.14).

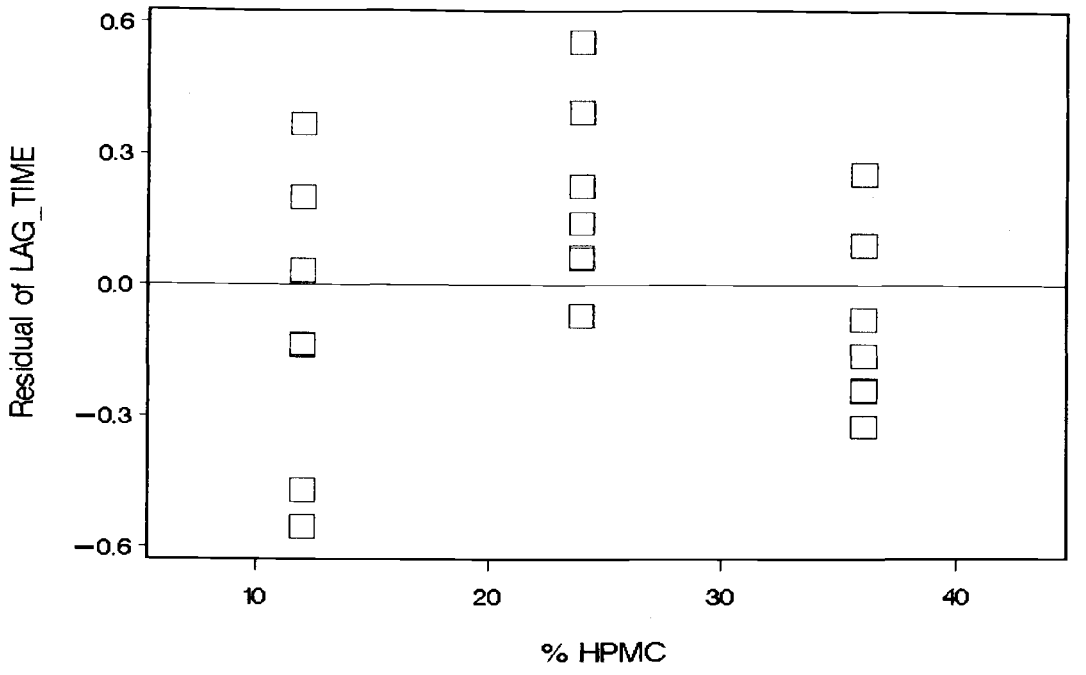


**Figure A.12: The plot of lag time residual with respect to % HPMC (non-random pattern indicates missing of %HPMC$^2$ term).**

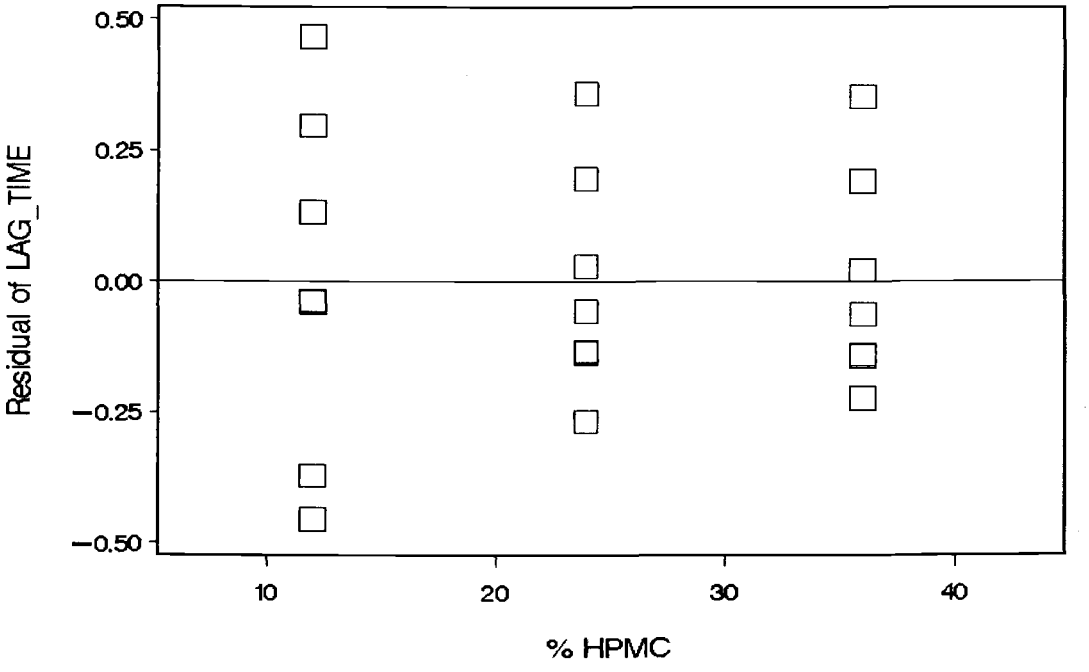


**Figure A.13: The plot of lag time residual with respect to % HPMC (random distribution with addition of HPMC$^2$).**

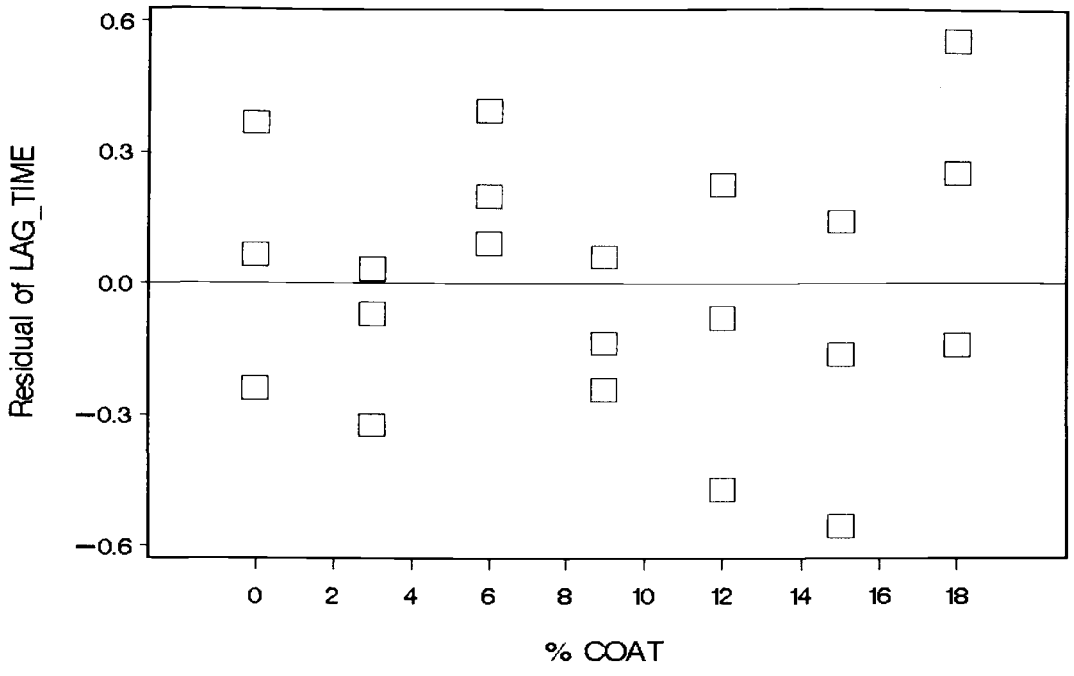


**Figure A.14: The plot of lag time residual with respect to % COAT (random distribution).**

Analysis of variance table for the regression model fit with time effect indicated that there is a significant difference among tablets prepared at different times (p-value <.0001, Table A.4).

**Table A.4: Analysis of variance table for lag time with time effect**

| Source | Sum of Squares | d.f. | Mean Square | F-Statistic | *p*-Value |
|---|---|---|---|---|---|
| % HPMC | 3.87053571 | 1 | 3.87053571 | 57.72 | <.0001 |
| % COAT | 86.10035714 | 1 | 86.10035714 | 1283.91 | <.0001 |
| Time | 0.90107143 | 1 | 0.90107143 | 13.44 | 0.0005 |
| Residual | 3.95660714 | 59 | 0.06706114 | | |
| Total | 94.82857143 | 62 | | | |

***R*-squared** = 95.83% **Estimated SD** = 0.258962

Regression model with a fixed effect of time

The plot of residual lag time versus % COAT demonstrated a random distribution of data which indicated that lack of fit was accommodated by the addition of time effects in the regression model (Figure A.15). The plot of the residual lag time versus % HPMC (Figure A.16) indicated a lack of fit that can be solved by adding the non-linear term of %HPMC (% $HPMC^2$, Figure A.17).

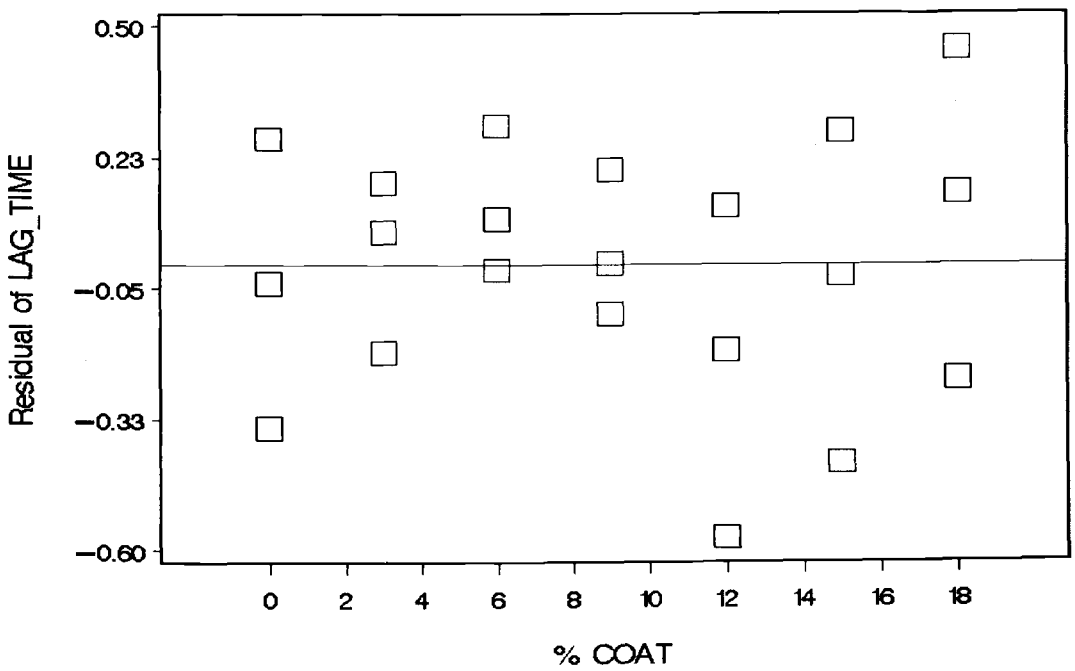


**Figure A.15: The plot of lag time residual with respect to % COAT after accounting for the fixed effect of time (random distribution).**

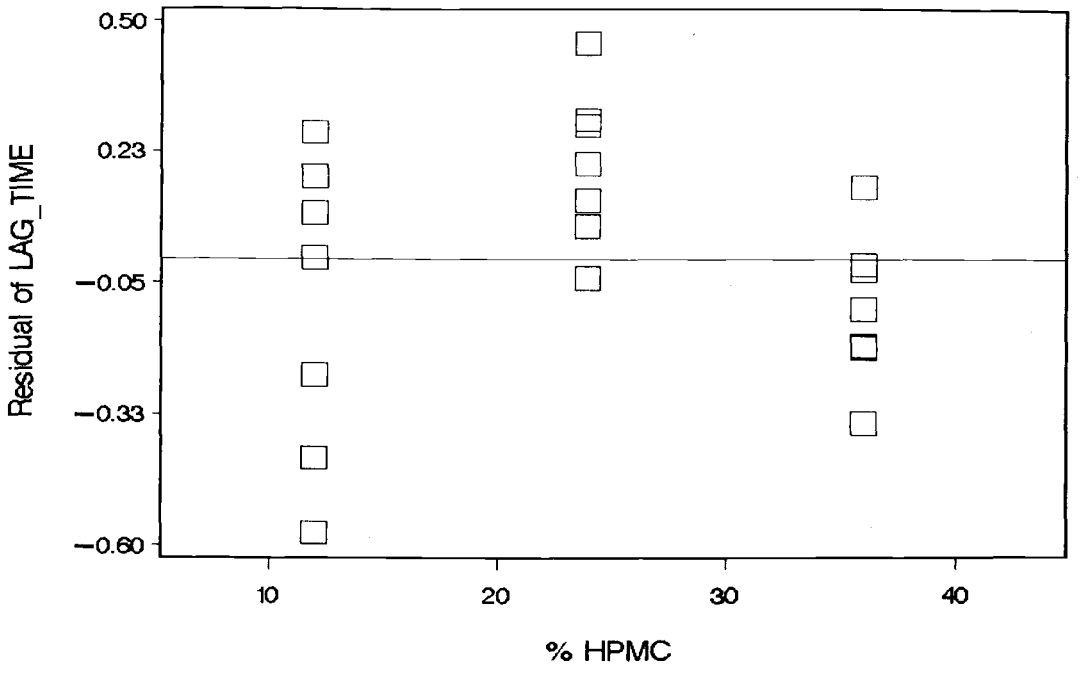


**Figure A.16: The plot of lag time residual with respect to % HPMC after accounting for the fixed effect of time (non-random distribution).**

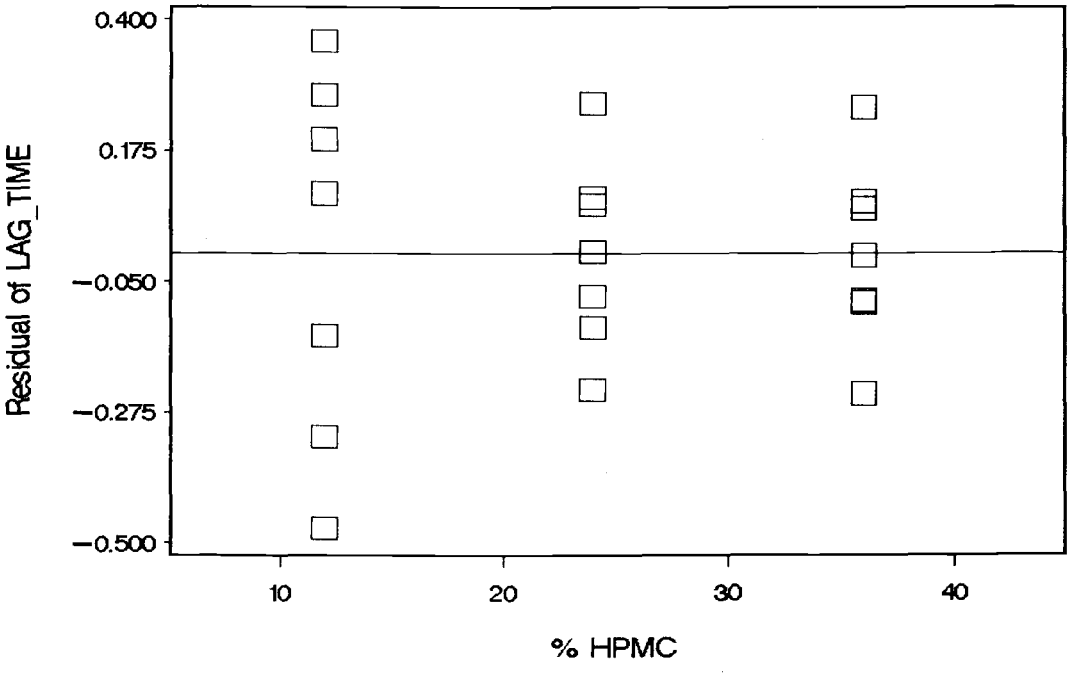


**Figure A.17: The plot of lag time residual with respect to % HPMC after accounting for the fixed effect of time (random distribution with additional of % HPMC$^2$).**

Residual plot from fitting the tentative model with and without the time effect showed a constant variance of data and suggested no need for transformation (Figure A.18). The tentative regression model of lag time as a function of explanatory variables is presented in equation 3-4.

Model without time effect

$$\mu\{\text{Lag time} \mid \text{hpmc, coat}\} = \text{hpmc} + \text{coat} + (\text{hpmc} \times \text{coat}) + \text{hpmc}^2 \qquad \text{equation (3)}$$

Model with time effect

$$\mu\{\text{Lag time} \mid \text{hpmc, coat}\} = \text{hpmc} + \text{coat} + (\text{hpmc} \times \text{coat}) + \text{hpmc}^2 + \text{time} \qquad \text{equation (4)}$$

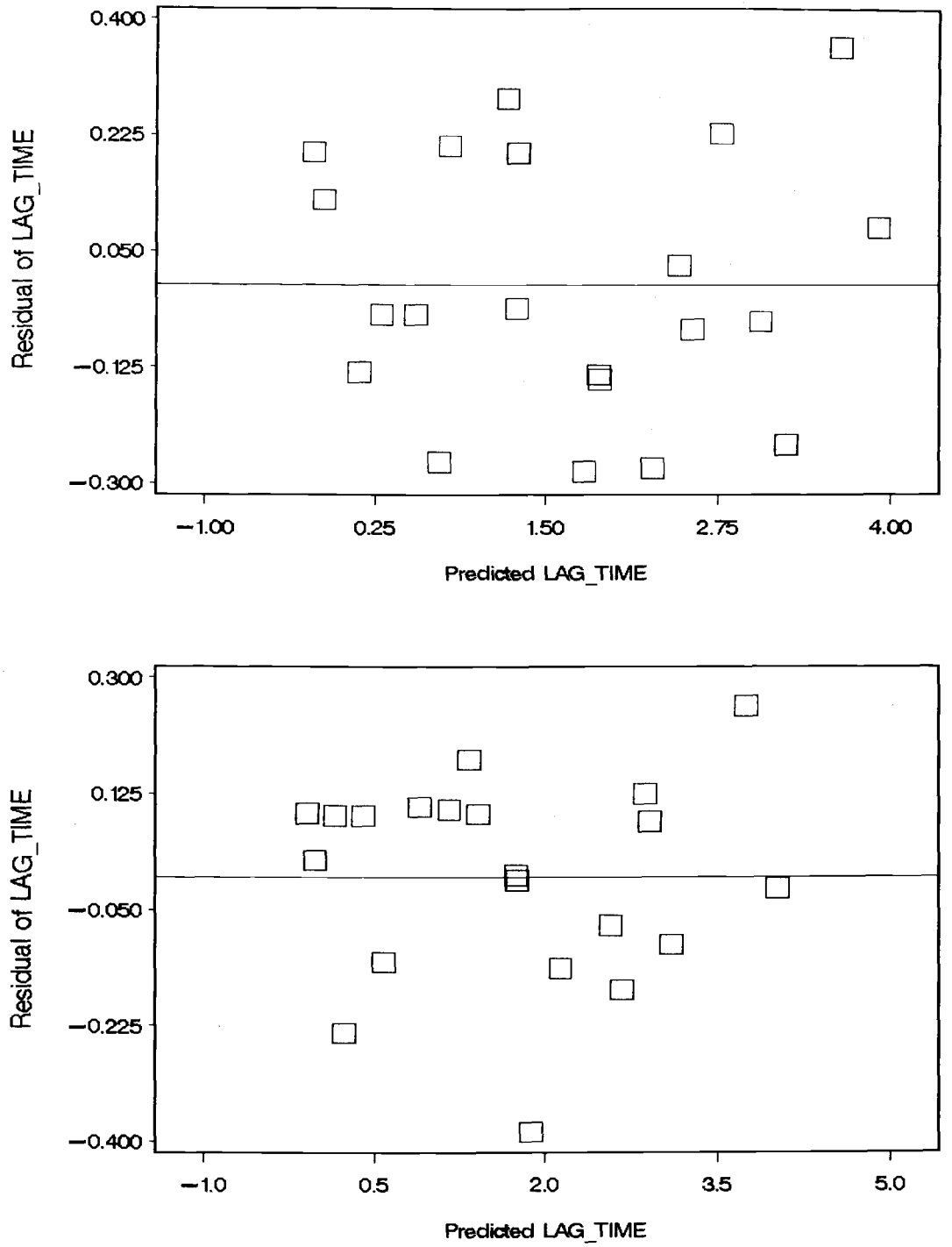


**Figure A.18: The plot of lag time residual versus its predicted value.** Panel A shows the residual plot without time effect; Panel B is the residual plot with the fixed effect of time.

**Data Exploration for Percent Drug Release at 6 Hours (DR 6 hrs) and 10 Hours (DR 10 hrs)**

A decrease in percent drug release at 6 hours and 10 hours is associated with an increase in coating amount, as determined by coded scatterplot (Figure A.20 and A.22). The plot also suggests a slight decrease in percent drug release at 6 hours and 10 hours when percent HPMC in the core tablet was increased (Figure A.19 and A.21). Type III test, however, showed that the amount of HPMC in the tablet has a significant effect on percent drug release at both 6 hours and 10 hours (Table A.5 and A.6).

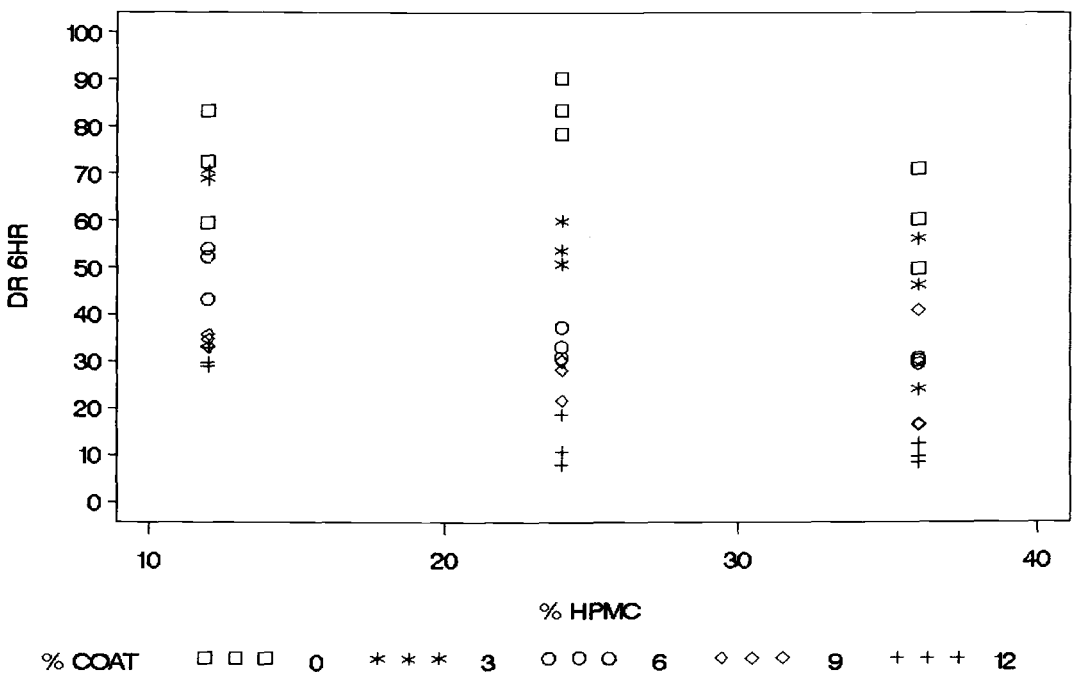


**Figure A.19: Coded scatterplot of DR 6 hrs versus %HPMC.**

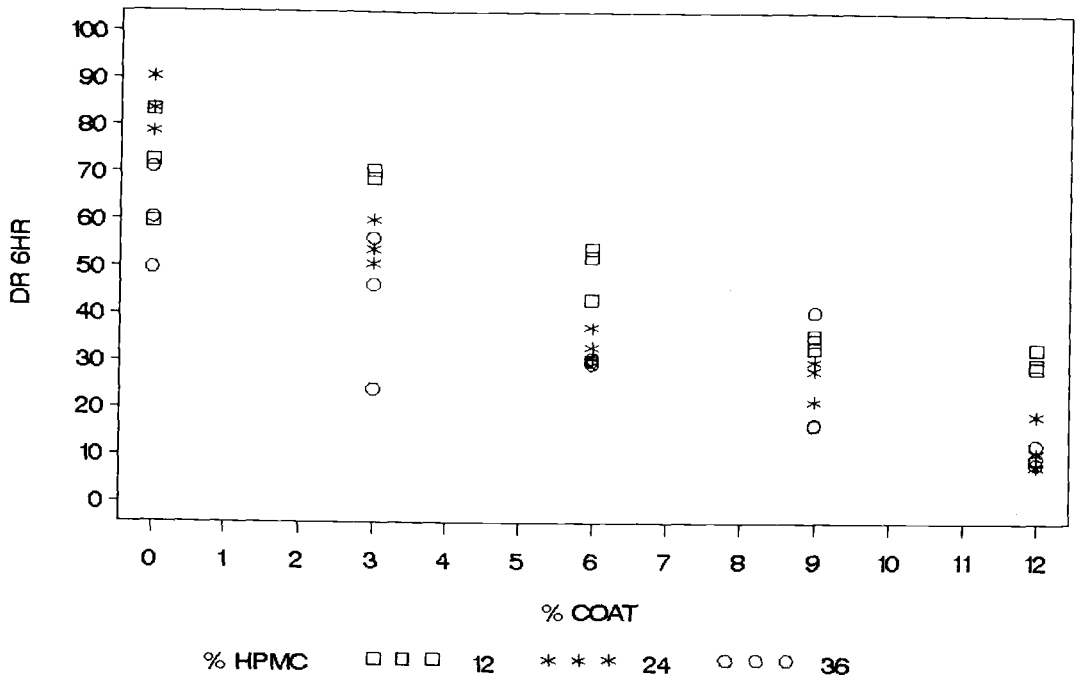


**Figure A.20: Coded scatterplot of DR 6 hrs versus %COAT**

**Table A.5: Type III test of % DR 6 hrs with respect to %HPMC and %COAT.**

| Source of Variation | DF | Type III SS | Mean Square | F-Value | Pr > F |
|---|---|---|---|---|---|
| %COAT | 4 | 16816.93036 | 4204.23259 | 71.44 | <.0001 |
| %HPMC | 2 | 2353.01119 | 1176.50560 | 19.99 | <.0001 |
| %HPMC * %COAT | 8 | 1179.14607 | 147.39326 | 2.50 | 0.0325 |

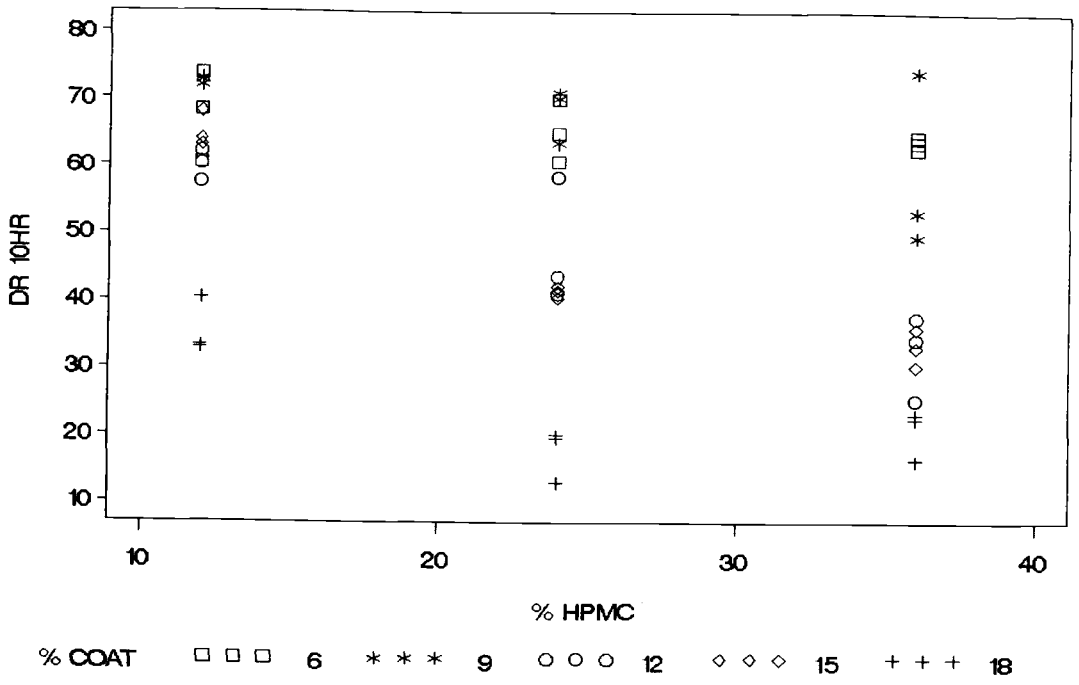


**Figure A.21: Coded scatterplot of DR 10 hrs versus%HPMC.**

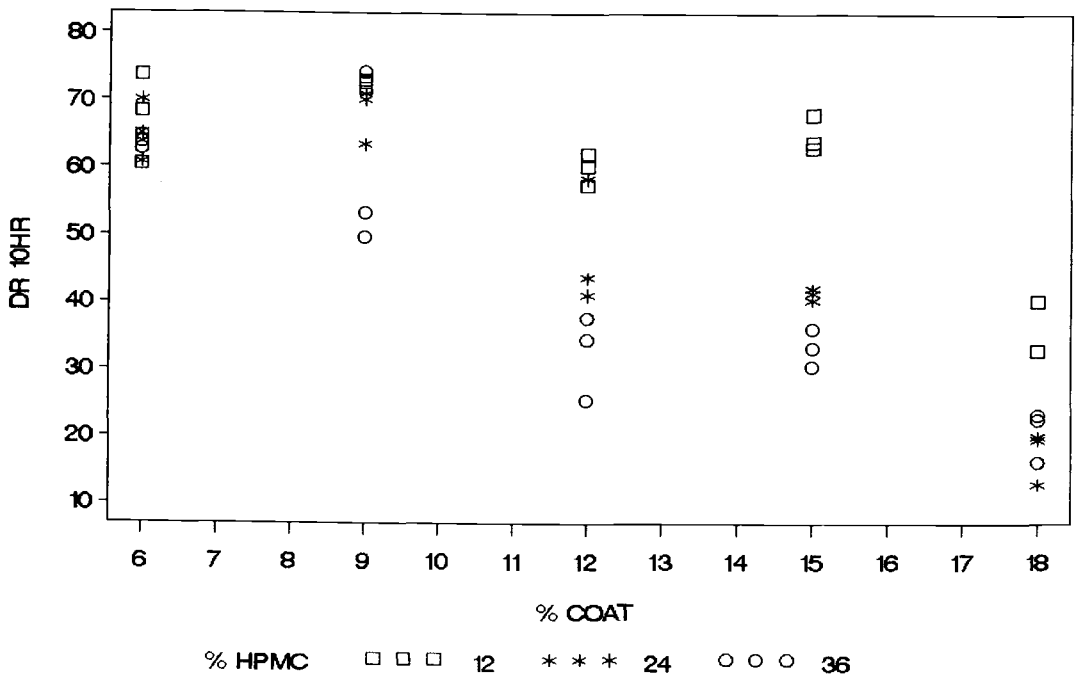


**Figure A.22: Coded scatterplot of DR 10 hrs versus % COAT.**

**Table A.6: Type III test of % DR 10 hrs with respect to %HPMC and %COAT**

| Source of Variation | DF | Type III SS | Mean Square | F-Value | Pr > F |
|---|---|---|---|---|---|
| %COAT | 4 | 10643.75868 | 2660.93967 | 87.47 | <.0001 |
| %HPMC | 2 | 2574.21482 | 1287.10741 | 42.31 | <.0001 |
| %HPMC * %COAT | 8 | 1035.26350 | 129.40794 | 4.25 | 0.0017 |

The plot of the residual of DR 6 hrs versus %HPMC (Figure A.23) and the residual of DR 10 hrs versus %HPMC (Figure A.25) showed a slight curvature pattern possibly due to missing a $\%HPMC^2$ term. Unfortunately, this curvature pattern could not be solved by additional of non-linear term ($\%HPMC^2$) in the regression model. Random distribution of data was observed with the plot of DR 6 hrs residual versus %COAT (Figure A.24). The plot of DR 10 hrs residual versus % COAT (Figure A.26) indicated a lack of fit. This pattern of lack of fit could not be solved by data transformation or the additional of a non-linear term. The non-random pattern observed from the residual plot of DR 6 hrs versus % HPMC, DR 10 hrs versus % HPMC, and DR 10 hrs versus % COAT may be due to missing a time effect.

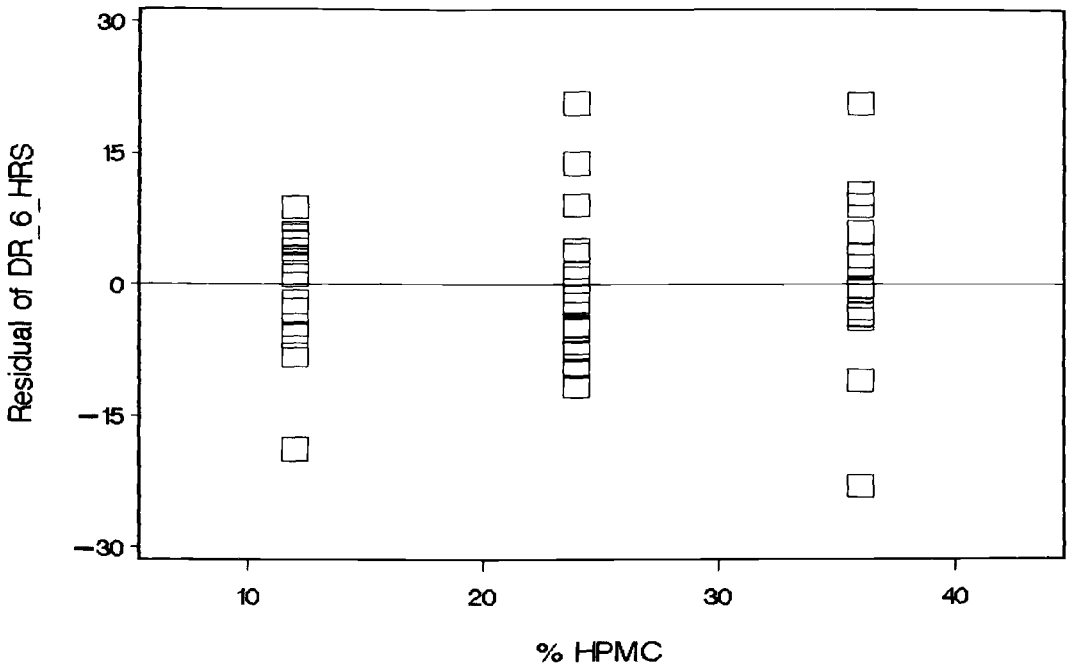


**Figure A.23: The plot of DR 6 hrs residual with respect to % HPMC.**

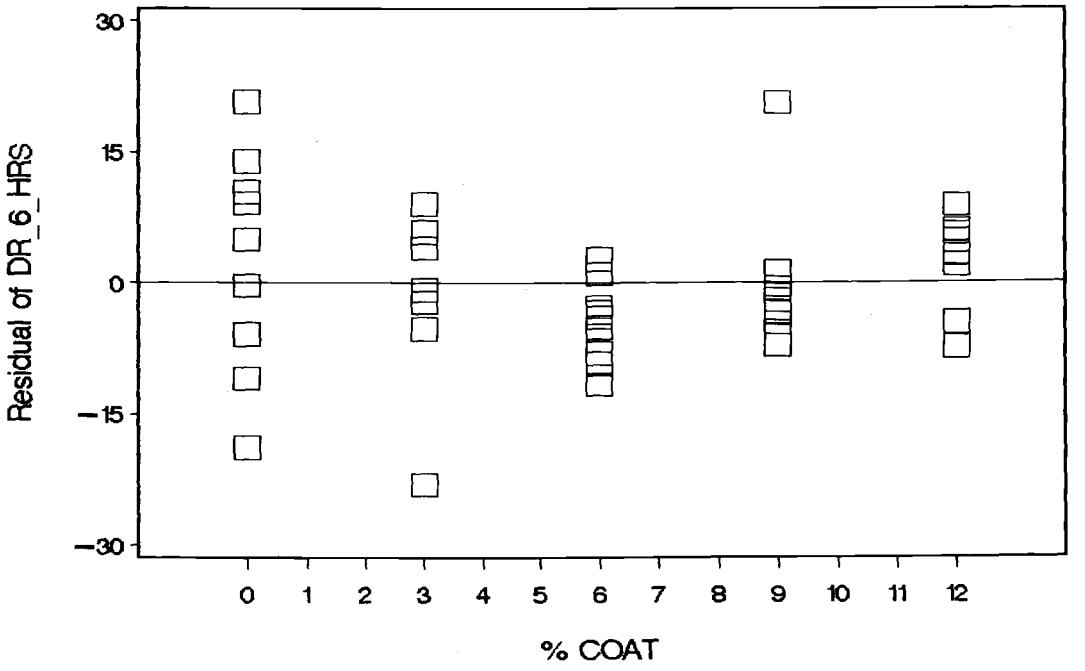


**Figure A.24: The plot of DR 6 hrs residual with respect to % COAT.**

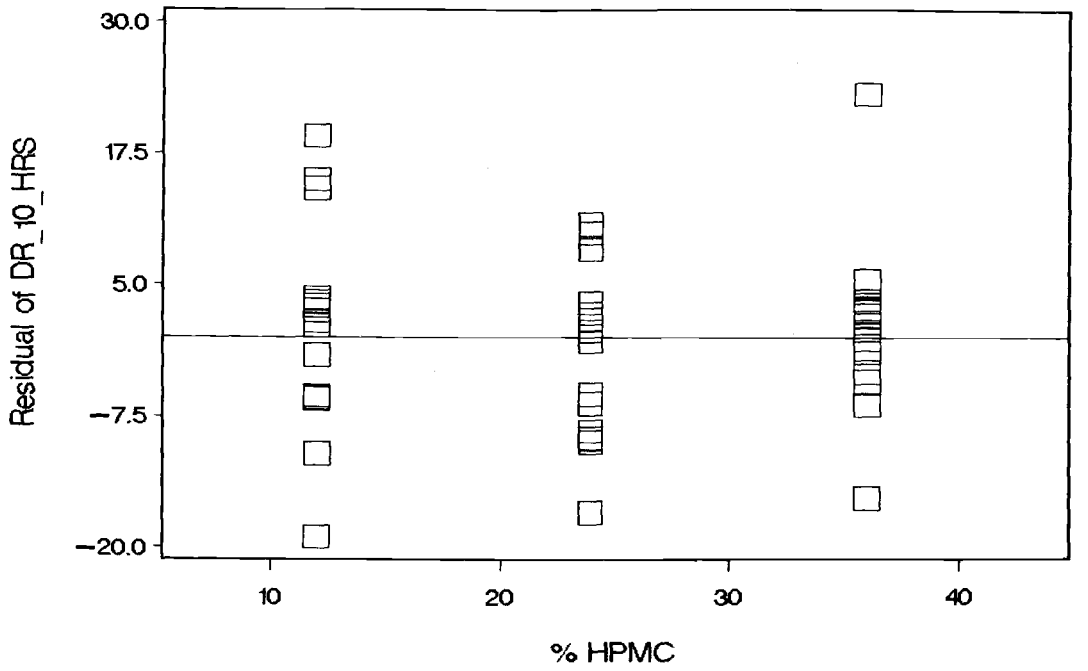


**Figure A.25: The plot of DR 10 hrs residual with respect to % HPMC.**

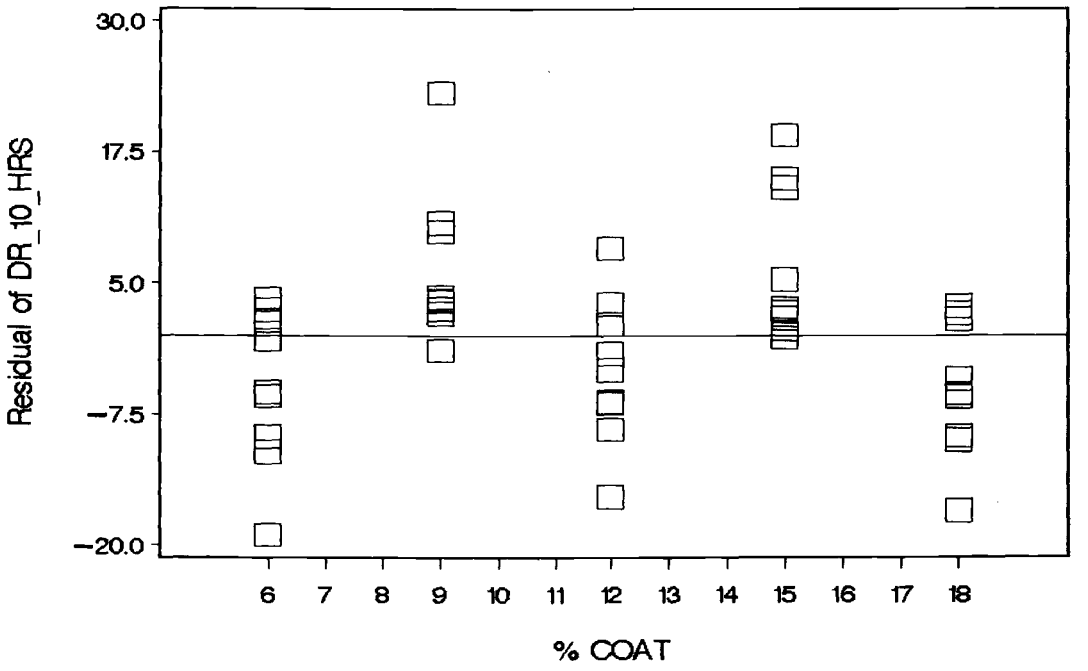


**Figure A.26: The plot of DR 10 hrs residual with respect to % COAT.**

Analysis of variance table for time effect on DR 6 hrs indicated that no significant difference among tablets prepared at different time periods (p-value <0.8406, Table A.7). While analysis of variance table for time effect on DR 10 hrs shows that there is a significant difference among tablets prepared on different time period (p-value <0.8406, Table A.8).

**Table A.7: Analysis of variance table for DR 6 hrs with time effect**

| Source | Sum of Squares | d.f. | Mean Square | F-Statistic | *p*-Value |
|---|---|---|---|---|---|
| % HPMC | 2353.00902 | 1 | 2353.00902 | 29.80 | <.0001 |
| % COAT | 16520.78147 | 1 | 16520.78147 | 209.21 | <.0001 |
| Time | 3.23637 | 1 | 3.23637 | 0.04 | 0.8406 |
| Residual | 3237.64882 | 41 | 78.96704 | | |
| Total | 22114.67568 | 44 | | | |

***R*-squared** = 85.36% **Estimated SD** = 8.886340

**Table A.8: Analysis of variance table for DR 10 hrs with time effect**

| Source | Sum of Squares | d.f. | Mean Square | F-Statistic | *p*-Value |
|---|---|---|---|---|---|
| % HPMC | 2485.912235 | 1 | 2485.912235 | 49.62 | <.0001 |
| % COAT | 9329.554028 | 1 | 9329.554028 | 186.23 | <.0001 |
| Time | 1296.460820 | 1 | 1296.460820 | 25.88 | <.0001 |
| Residual | 2053.99155 | 41 | 50.09735 | | |
| Total | 15165.91863 | 44 | | | |

***R*-squared** = 86.46% **Estimated SD** = 7.077948

Regression model with a fixed effect of time

The plot of residual DR 10 hrs versus % COAT demonstrated a slightly curvature pattern (Figure A.27) which can be solved by additional of %COAT$^2$ term in the regression model (Figure A.28). The plot of residual DR 10 hrs versus % HPMC after including time effect showed a random distribution of data (Figure A.29) which suggests that lack of fit is caused by the missing of time effect.

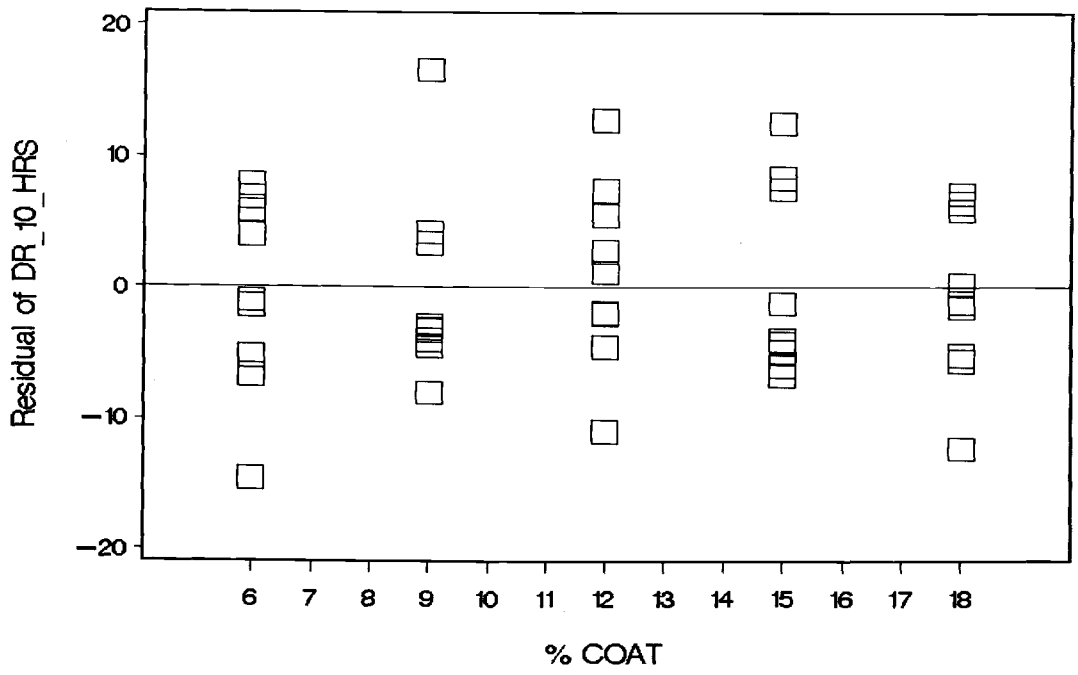


**Figure A.27: The plot of DR 10 hrs residual with respect to % COAT after accounting for the fixed effect of time (non-random distribution).**

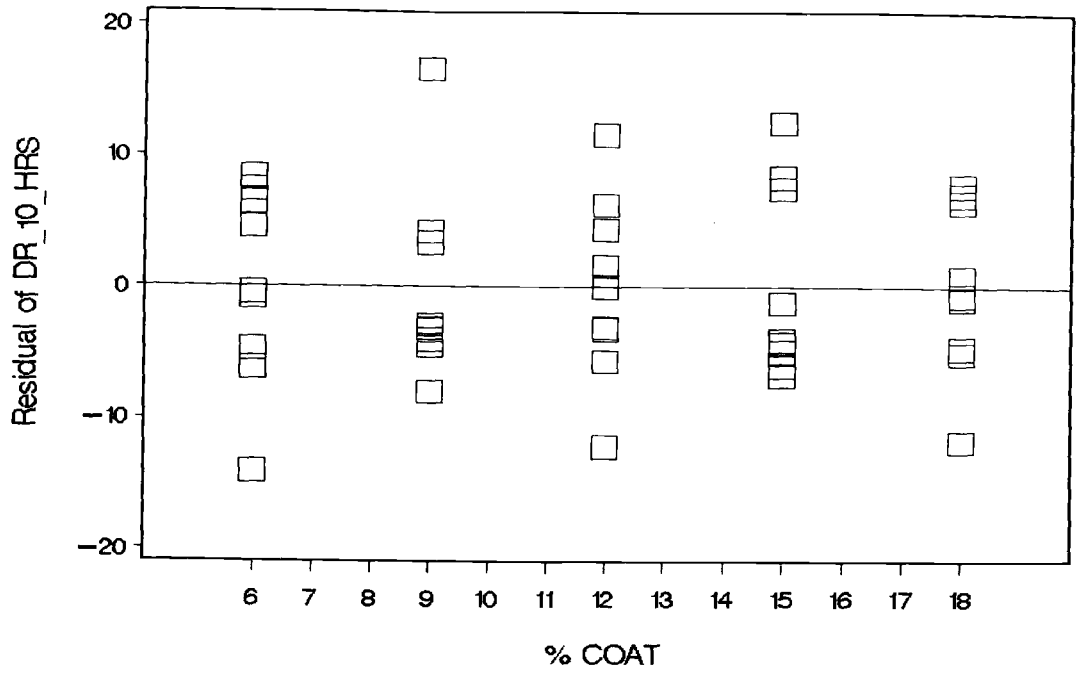


**Figure A.28: The plot of DR 10 hrs residual with respect to % COAT after accounting for the fixed effect of time (random distribution with additional of %COAT$^2$).**

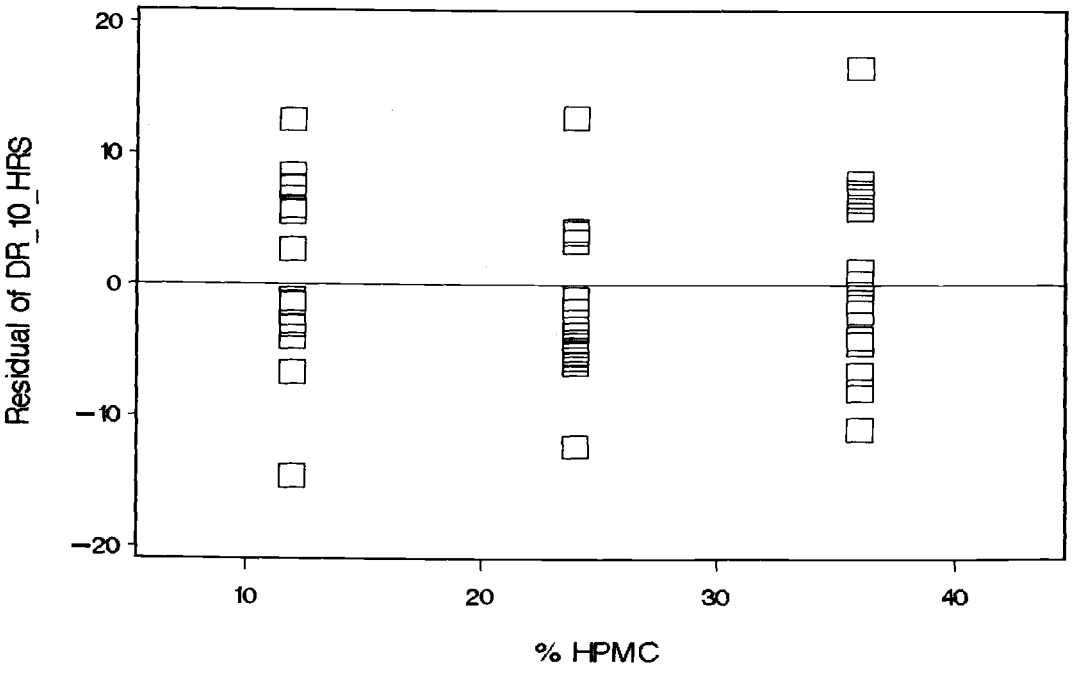


**Figure A.29: The plot of DR 10 hrs residual with respect to % HPMC after accounting for the fixed effect of time (random distribution).**

The tentative regression model of percent drug release at 6 and 10 hours as a function of explanatory variables is presented in equation 5-7. Residual plot obtained from the fit of regression model with and without time effect showed that data possesses a constant variance (Figure A.30 and A.31).

Model without time effect

$$\mu\{DR6hrs \mid hpmc, coat\} = hpmc + coat + (hpmc \times coat) + coat^2 \qquad \text{equation (5)}$$

$$\mu\{DR10hrs \mid hpmc, coat\} = hpmc + coat + (hpmc \times coat) + coat^2 \qquad \text{equation (6)}$$

Model with time effect

$$\mu\{DR10hrs \mid hpmc, coat\} = hpmc + coat + (hpmc \times coat) + coat^2 + time \qquad \text{equation (7)}$$

Where, DR6hrs and DR10hrs is percent drug release at 6 and 10 hours, respectively. Note that the amount of coating used in fitting the regression model of percent drug release at 6 hours was 0%, 3%, 6%, 9%, and 12%. Coating amount of 6%, 9%, 12%, 15%, and 18% was used in fitting the regression model of drug release at 10 hours.

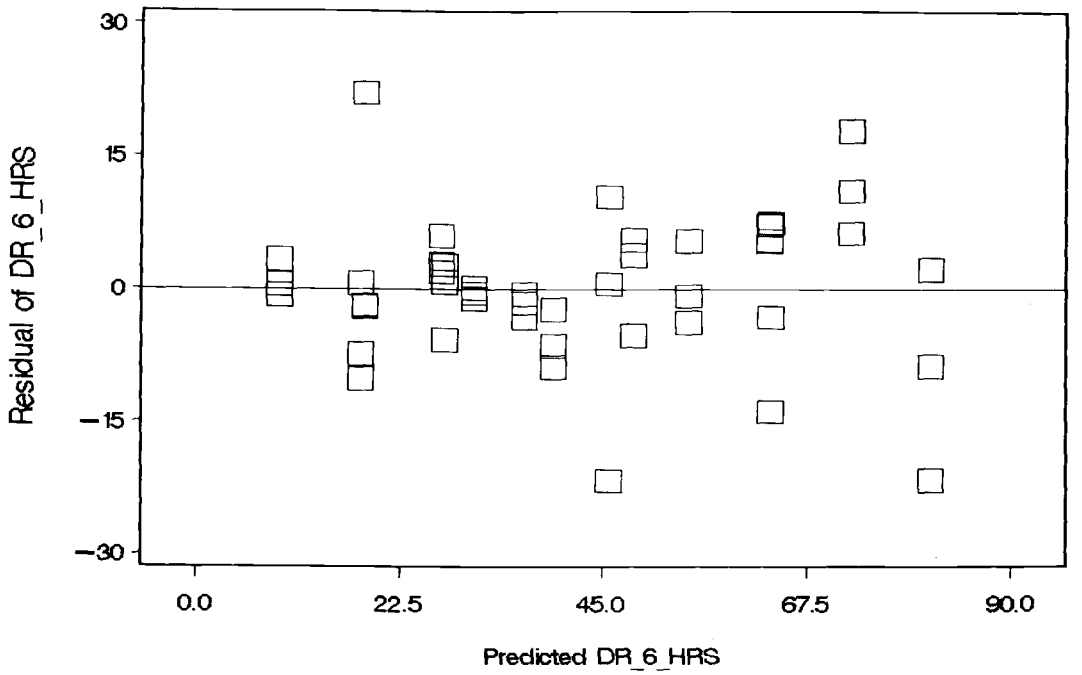


**Figure A.30: The plot of DR 6 hrs residual versus its predicted value (without time effect).**

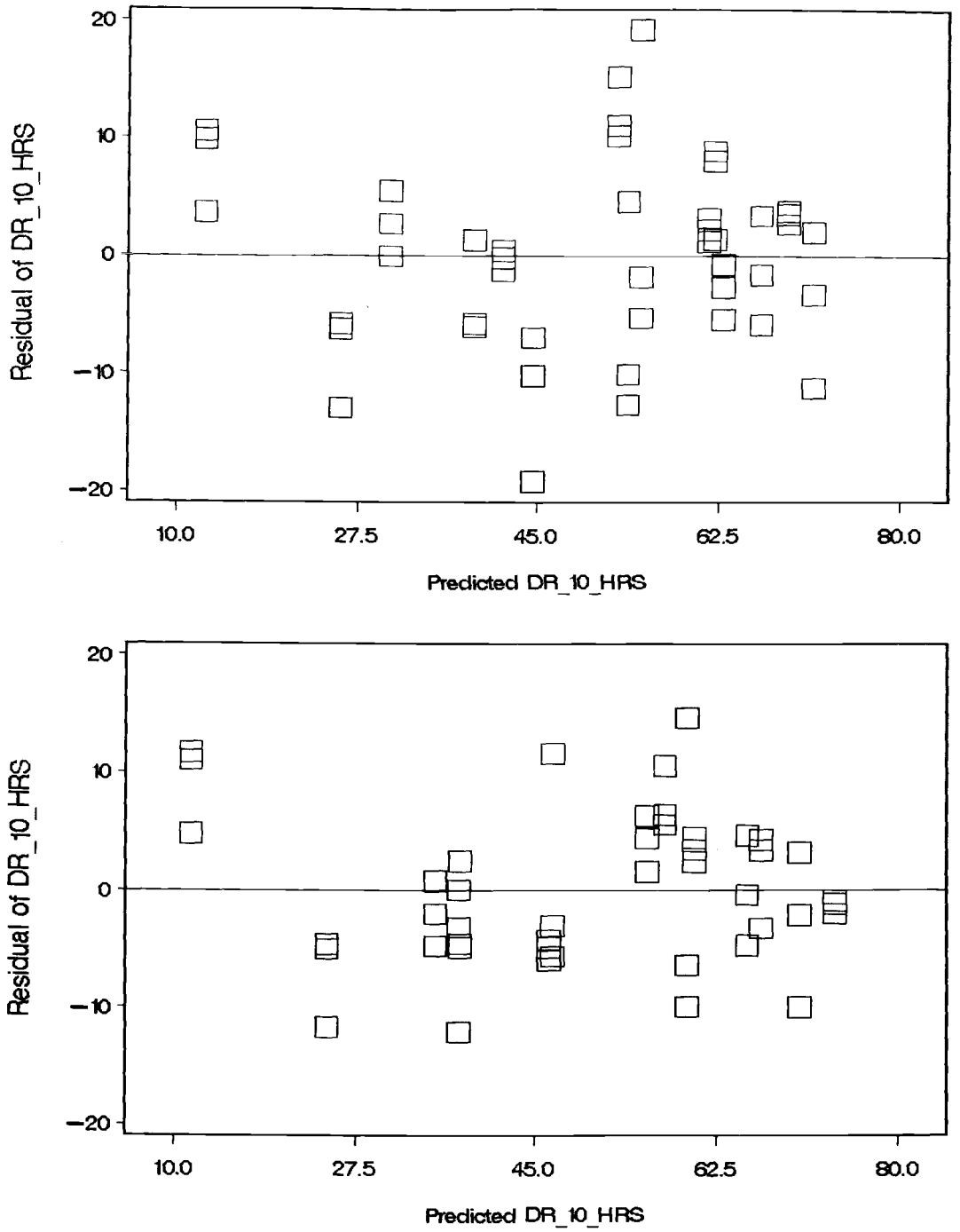


**Figure A.31: The plot of DR 10 hrs residual versus its predicted value.** Panel A shows the residual plot without time effect; Panel B is the residual plot with the fixed effect of time.

## Regression Analysis

Multiple linear regressions were performed using mixed linear model to find the best fit model for predicting percent cyclobenzaprine HCL release from matrix tablets. Mathematical equations generated from regression analysis of n-value, lag time, and percent drug release as a function of the amount of hydrophilic material (%HPMC) and coating amount (%COAT) after accounting for the fixed effect of time period (time 1 or time 2) were investigated. The time variable is defined as a class variable and the quantitative variables are % HPMC and % COAT. Mathematical model equations for predicting n-value, lag time, and percent drug release at 6 and 10 hours along with their analysis of variance table and parameter estimates including variable coefficient, standard error, t-statistic, and p-value are shown in equation 8-11 and Table A.9-A.16.

## N-value

$$\text{N-value} = 0.6529 + 0.01101\ \text{hpmc} + 0.09333\ \text{coat} - 0.00330\ \text{coat}^2 - 0.1833\ \text{time} \qquad \text{equation (8)}$$

Let; time = 0 for tablet formulation prepared at time 1 period

time = 1 for tablet formulation prepared at time 2 period

**Table A.9: Analysis of variance table for n-value**

| Source | Sum of Square | d.f. | Mean Squares | *F*-Statistics | *p*-value |
|---|---|---|---|---|---|
| Regression | 4.9219 | 4 | 1.2305 | 42.03 | <0.0001 |
| Residual | 1.6978 | 58 | 0.0293 | | |
| Total | 6.6197 | 62 | | | |

**Table A.10: Parameter estimates table for n-value**

| Variables | Coefficient Estimate | SE | t-stat | Pr >\|t\| |
|---|---|---|---|---|
| Intercept | 0.6529 | 0.08164 | 8.00 | <0.0001 |
| HPMC | 0.01101 | 0.0022 | 5.00 | <.0001 |
| COAT | 0.09333 | 0.01368 | 6.82 | <.0001 |
| $COAT^2$ | -0.00330 | 0.000733 | -4.50 | <.0001 |
| Time 1 | -0.1833 | 0.04620 | -3.97 | 0.0002 |

**Lag time**

Lag time = $-1.2631 + 0.1018\ hpmc + 0.1353\ coat + 0.002480\ (hpmc \times coat)$

$-0.0021\ hpmc^2 + 0.2417\ (time1)$ equation (9)

**Table A.11: Analysis of variance table for lag time**

| Source | Sum of Square | d.f. | Mean Squares | *F*-Statistics | *p*-value |
|---|---|---|---|---|---|
| Regression | 93.4414 | 5 | 18.6883 | 767.93 | <0.0001 |
| Residual | 1.3871 | 57 | 0.0243 | | |
| Total | 94.8286 | 62 | | | |

**Table A.12: Parameter estimates table for lag time**

| Variables | Coefficient Estimate | SE | t-stat | Pr > \|t\| |
|---|---|---|---|---|
| Intercept | -1.2631 | 0.1692 | -7.47 | <0.0001 |
| HPMC | 0.1018 | 0.01436 | 7.09 | <0.0001 |
| COAT | 0.1353 | 0.008667 | 15.61 | <0.0001 |
| HPMC×COAT | 0.00248 | 0.000334 | 7.42 | <0.0001 |
| $HPMC^2$ | -0.00206 | 0.00029 | -7.11 | <0.0001 |
| Time 1 | 0.2417 | 0.03972 | 6.08 | <0.0001 |

**Percent Drug Release at 6 Hours (% DR 6 hrs)**

There is no significant difference in DR 6 hrs among tablets prepared at different time periods (p-value <0.8406). Thus model equation for DR 6 hrs was generated without time effect.

$$DR6hr = 90.0855 - 0.7380\ hpmc - 6.3939\ coat + 0.1565\ coat^2 \qquad \text{equation (10)}$$

**Table A.13: Analysis of variance table for percent drug release at 6 hours**

| Source | Sum of Square | d.f. | Mean Squares | *F*-Statistics | *p*-value |
|---|---|---|---|---|---|
| Regression | 19123.6678 | 3 | 6374.5559 | 87.38 | <0.0001 |
| Residual | 2991.0079 | 41 | 72.9514 | | |
| Total | 22114.6757 | 44 | | | |

**Table A.14: Parameter estimates table for percent drug release at 6 hours**

| Variables | Coefficient Estimate | SE | t-stat | Pr > \|t\| |
|---|---|---|---|---|
| Intercept | 90.0855 | 4.1117 | 21.91 | <0.0001 |
| HPMC | -0.7380 | 0.1299 | -5.68 | <0.0001 |
| COAT | -6.3939 | 1.0580 | -6.04 | <0.0001 |
| $COAT^2$ | 0.1565 | 0.0845 | 1.85 | 0.0714 |

**Percent Drug Release at 10 Hours (% DR 10 hrs)**

$DR10hr = 96.2009 - 0.7586\ hpmc\ -0.1399\ coat^2 - 8.8585\ (time\ 1)$ equation (11)

**Table A.15: Analysis of variance table for percent drug release at 10 hours**

| Source | Sum of Square | d.f. | Mean Squares | *F*-Statistics | *p*-value |
|---|---|---|---|---|---|
| Regression | 13060.8142 | 3 | 4353.6047 | 84.79 | <0.0001 |
| Residual | 2105.1044 | 41 | 51.3440 | | |
| Total | 15165.9186 | 44 | | | |

**Table A.16: Parameter estimates table for percent drug release at 10 hours**

| Variables | Coefficient Estimate | SE | t-stat | Pr >\|t\| |
|---|---|---|---|---|
| Intercept | 96.2009 | 3.4975 | 27.51 | <0.0001 |
| HPMC | -0.7586 | 0.1090 | -6.96 | <0.0001 |
| $COAT^2$ | -0.1399 | 0.01040 | -13.44 | <0.0001 |
| Time 1 | -8.8585 | 2.1860 | -4.05 | 0.0002 |

**Model Validation**

Predicted drug release profile of tablets prepared with 30% HPMC at 0%, 3%, 6%, and 9% coating at time 1 (predicted time 1) demonstrated a similar correlation to the predicted drug release profile of tablets prepared with 30% HPMC at 0%, 3%, 6%, and 9% coating at time 2 (predicted time 2) (Figure A.32-A.35). Lack of fit was observed with dosage form prepared with 30% HPMC at 12%, 15%, and 18% coating (Figure A.36-A.38). The deviation of predicted release profiles from the observed values was more sensitive at higher amounts of coating.

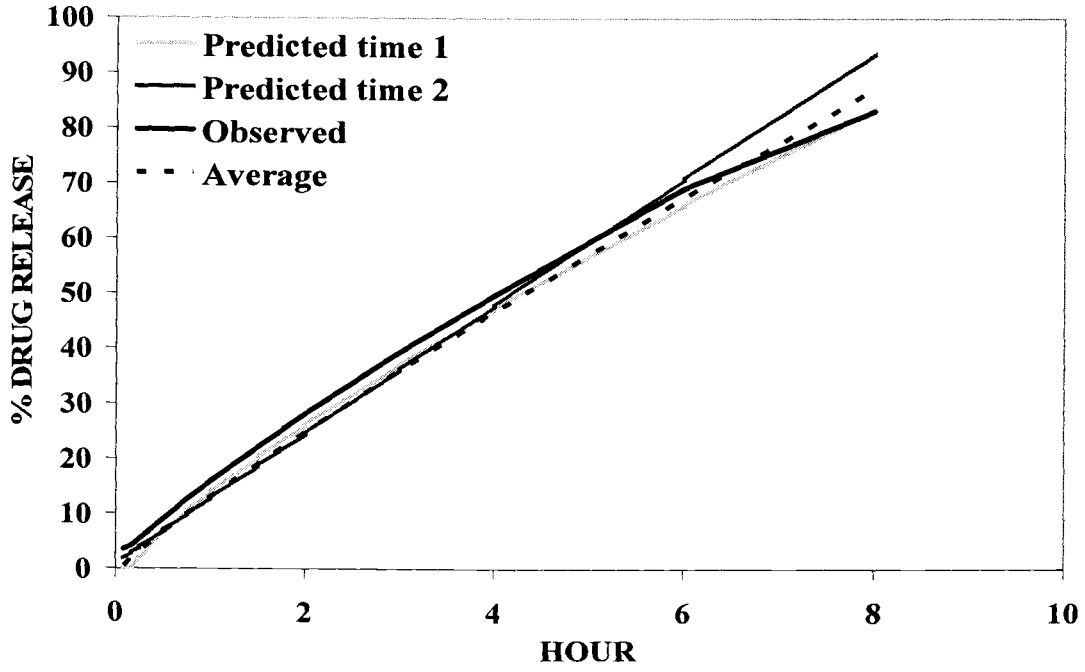


**Figure A.32: Release of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 0% coating.**

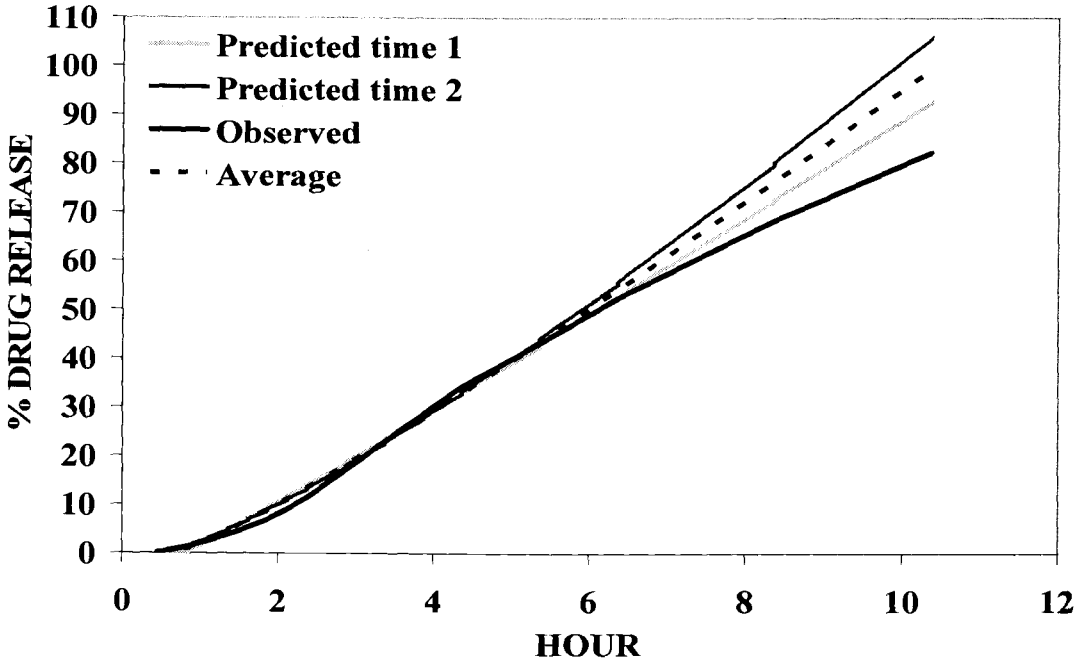


**Figure A.33: Release of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 3% coating.**

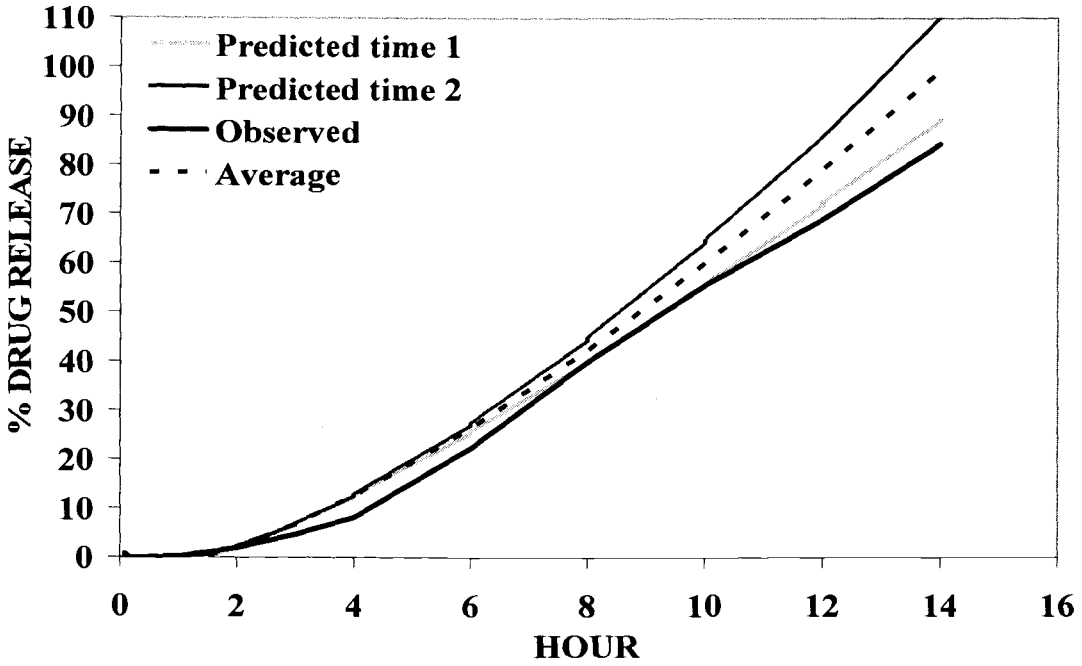


**Figure A.34: Release of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 6% coating.**

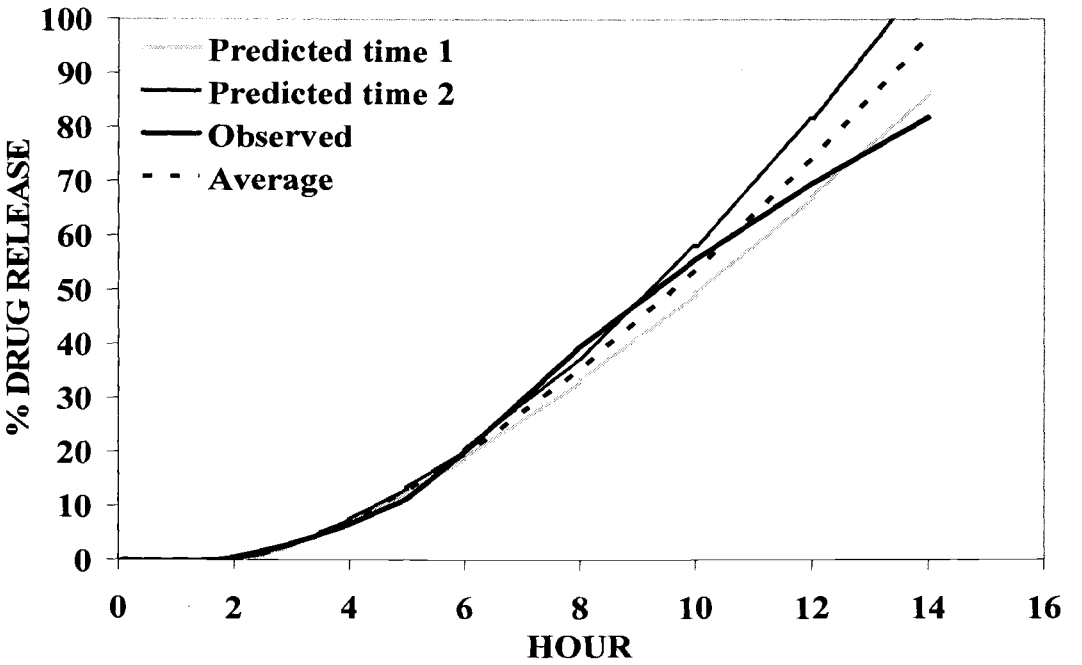


**Figure A.35: Release of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 9% coating.**

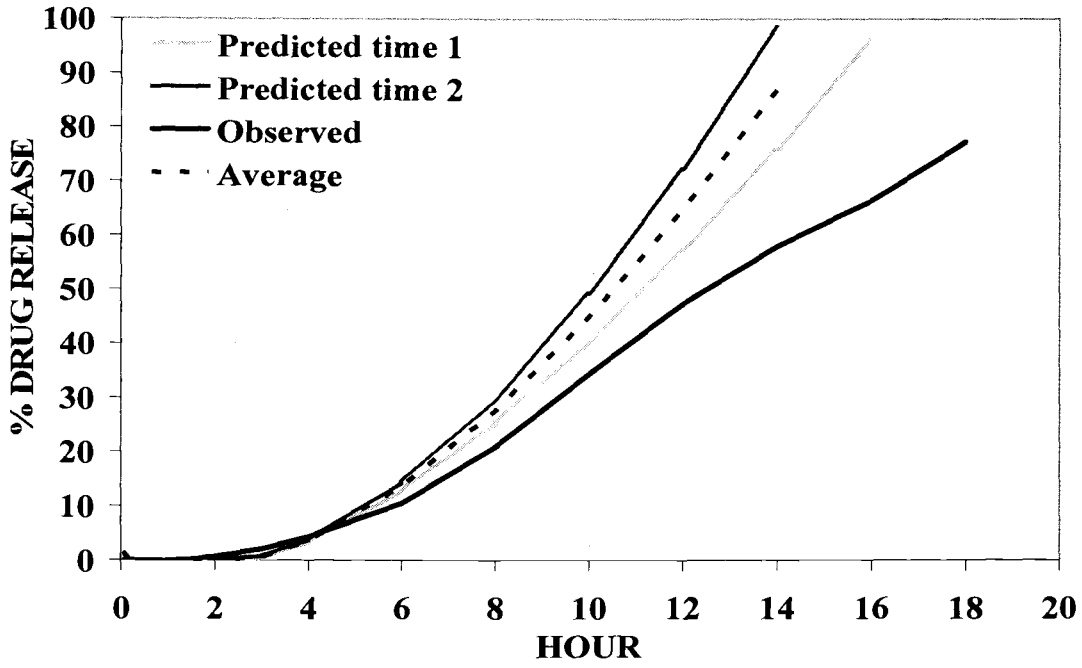


**Figure A.36: Release of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 12% coating.**

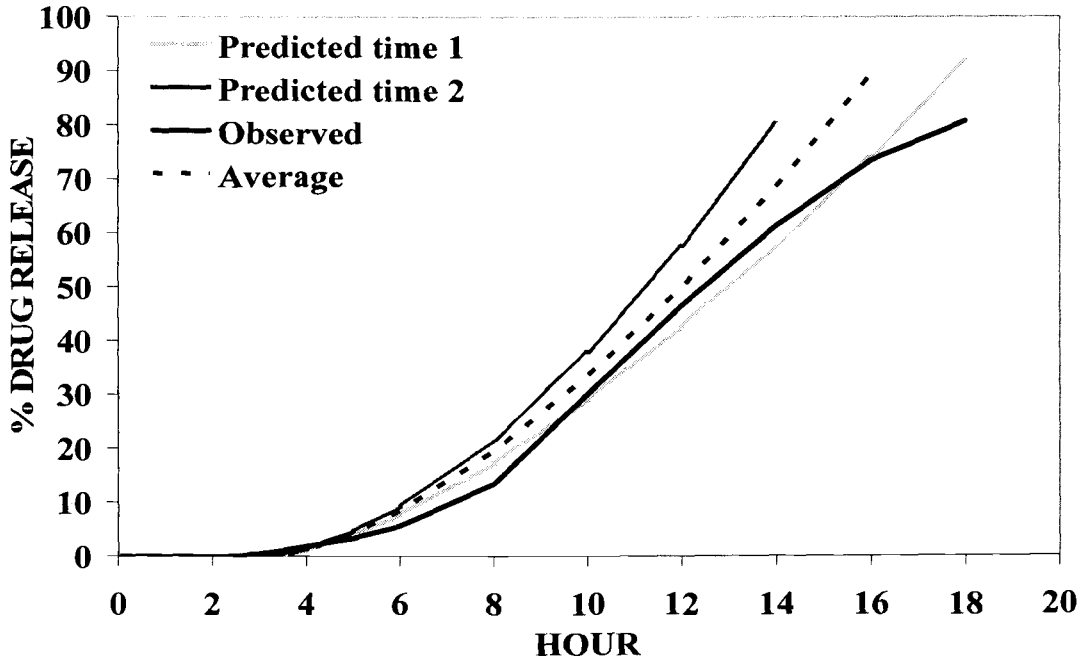


**Figure A.37: Release of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 15% coating.**

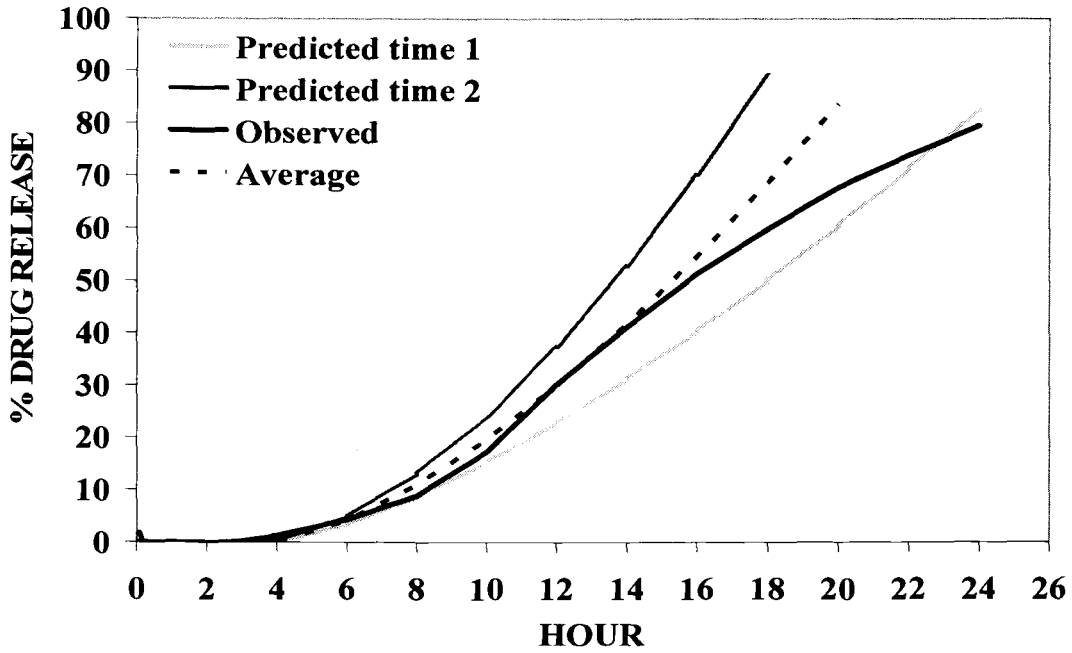


**Figure A.38: Release of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 18% coating.**

Comparison of two predicted *in vitro* drug release profiles (dissolution profile) was also performed using convolution method. Convolution method was used to convert the dissolution profile to a plasma concentration time profile. According to FDA guidance for bioequivalence study, two dosage formulations that have differences in maximum concentration (Cmax) of equal to or less than 20% will not be clinically significantly different. Additional, the similarity factor ($f_2$) can also be calculated to determine whether two dissolution profiles are similar. Two dissolution profiles are considered to be similar when the value of $f_2$ is larger than 50.

$$f_2 = 50 \times \log\left\{\left[1+\frac{1}{n}\sum_{i=1}^{n}(R_i - T_i)^2\right]^{-0.5} \times 100\right. \qquad \text{equation (1)}$$

Where:

n = sample number

$R_i$ = percent dissolved of reference product at time i

$T_i$ = percent dissolved of test product at time i

Predicted plasma concentration time profile of tablets prepared with 30% HPMC at 0%, 3%, 6%, and 9% coating at time 1 (predicted time 1) demonstrated a similar correlation to the predicted plasma concentration time profile of tablets prepared with 30% HPMC at 0%, 3%, 6%, and 9% coating at time 2 (predicted time 2) (Figure A.39-A.42). The difference in maximum concentration (Cmax) is less than 20%. Additional, the similarity factor ($f_2$) shows that predicted drug release profile obtained from equation with time 1 is the same as predicted drug release profile obtained from equation with time 2 ($f_2 > 50$). Lack of fit was observed with dosage form prepared with 30% HPMC at 12%, 15%, and 18% coating (Figure A.43-A.45). The deviation of predicted release profile from the observed value was more sensitive at higher amount of coating. Predicted drug release profiles obtained from the average equation (average of time1 and time 2 equation) demonstrated a similar correlation with the observed drug release profiles in all tested formulation except for tablets prepared with 30% HPMC at 12% COAT ($f_2 = 42.58877$).

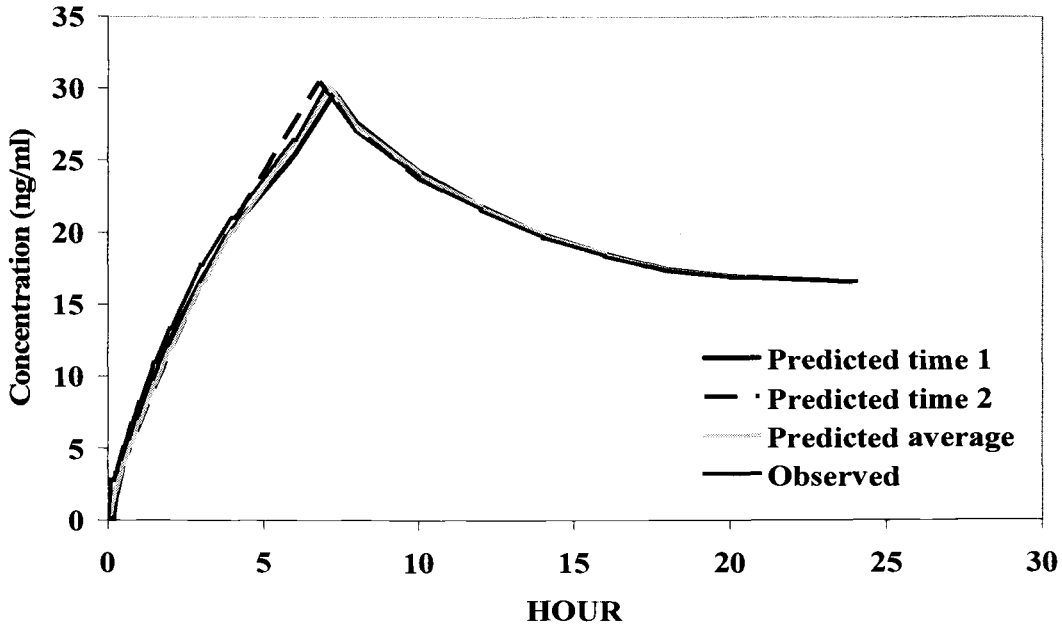


**Figure A.39: Convolution of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 0% coating.** Similarity factor 2 between observed and predicted average is 75.79589. Similarity factor 2 between predicted time 1 and time 2 is 72.15116.

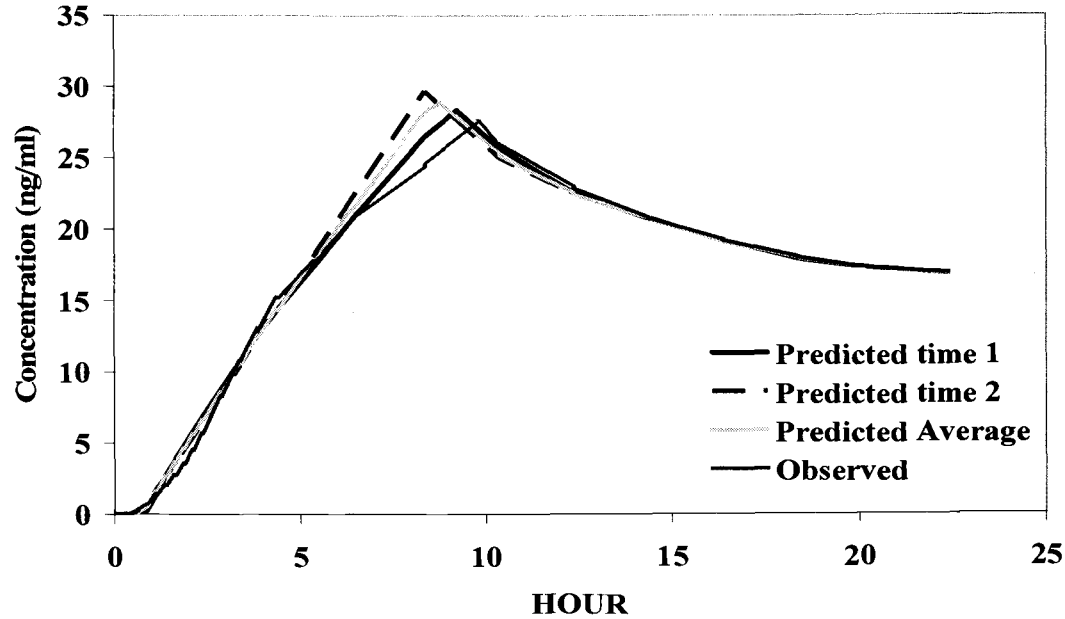


**Figure A.40: Convolution of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 3% coating.** Similarity factor 2 between observed and predicted average is 66.31908. Similarity factor 2 between predicted time 1 and time 2 is 70.78524.

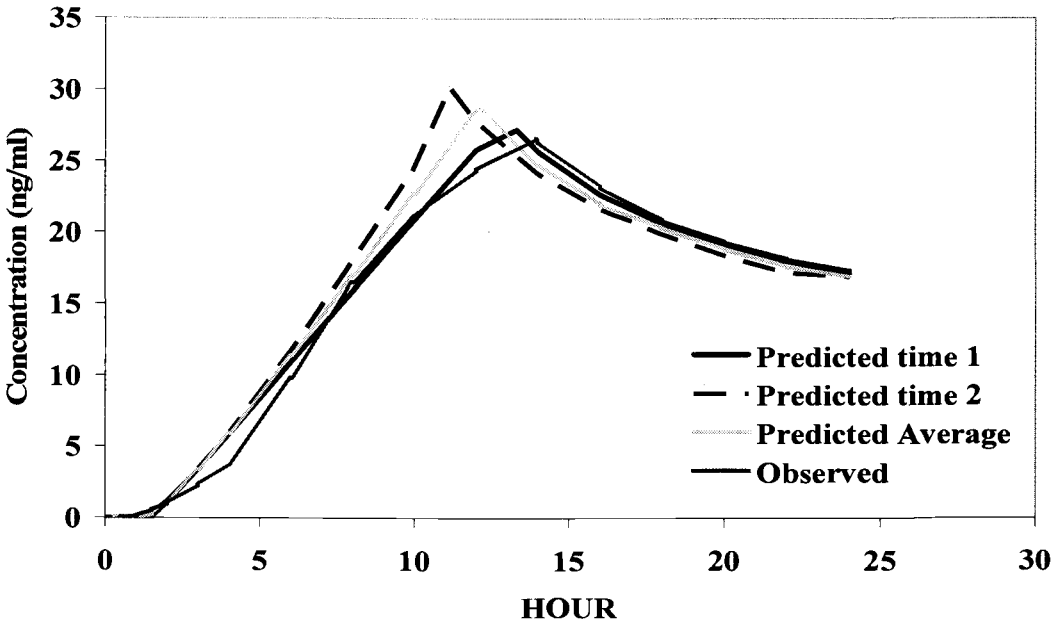


**Figure A.41: Convolution of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 6% coating.** Similarity factor 2 between observed and predicted average is 63.97156. Similarity factor 2 between predicted time 1 and time 2 is 57.74066.

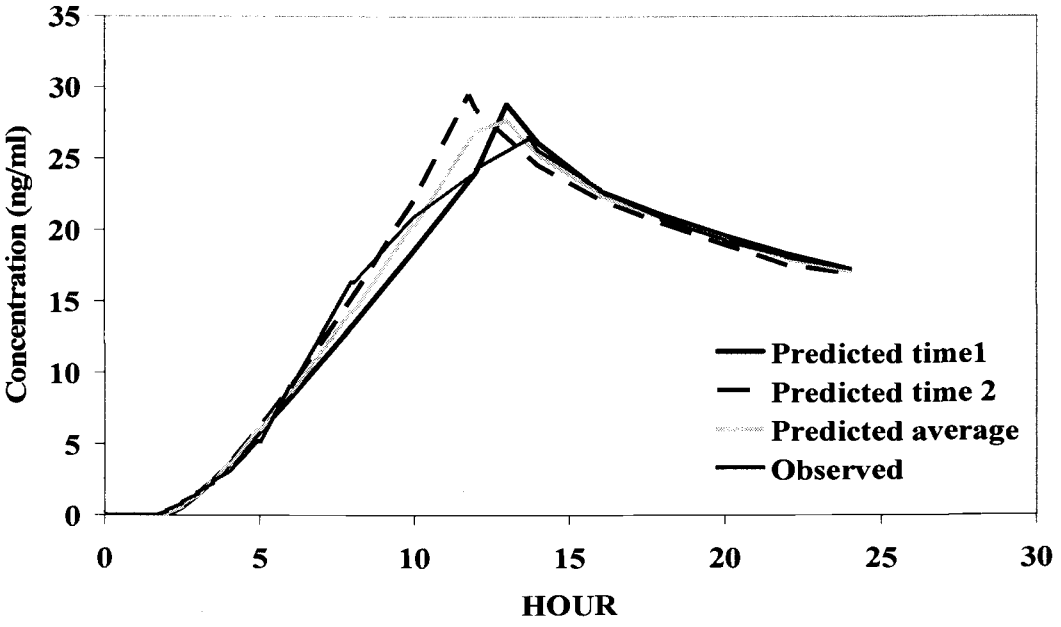


**Figure A.42: Convolution of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 9% coating.** Similarity factor 2 between observed and predicted average is 70.69089. Similarity factor 2 between predicted time 1 and time 2 is 59.83277.

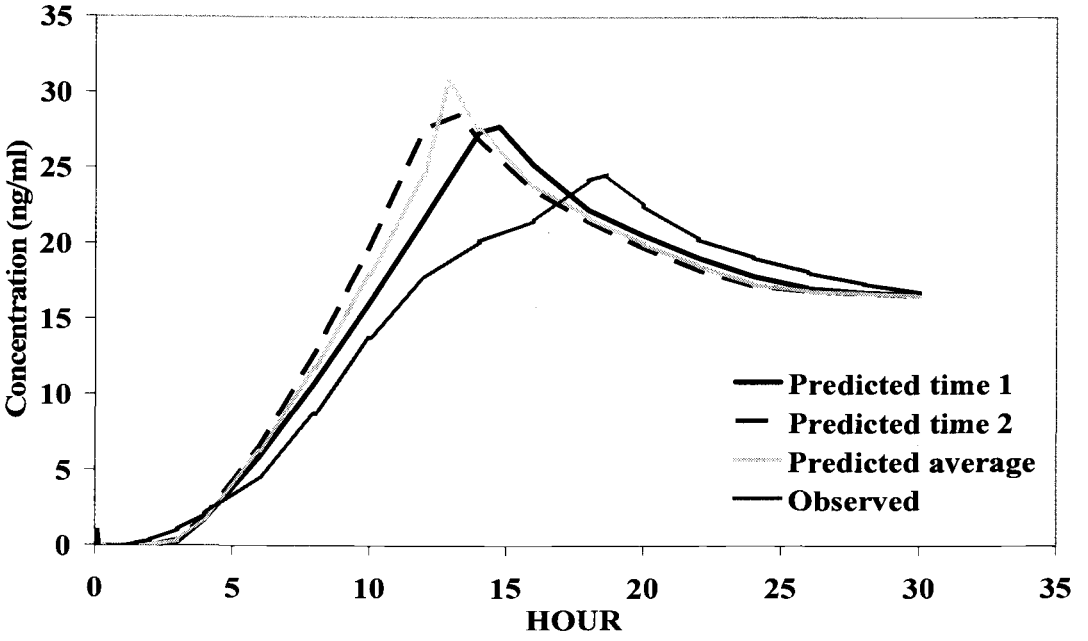


**Figure A.43: Convolution of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 12% coating.** Similarity factor 2 between observed and predicted average is 42.58877. Similarity factor 2 between predicted time 1 and time 2 is 56.22745.

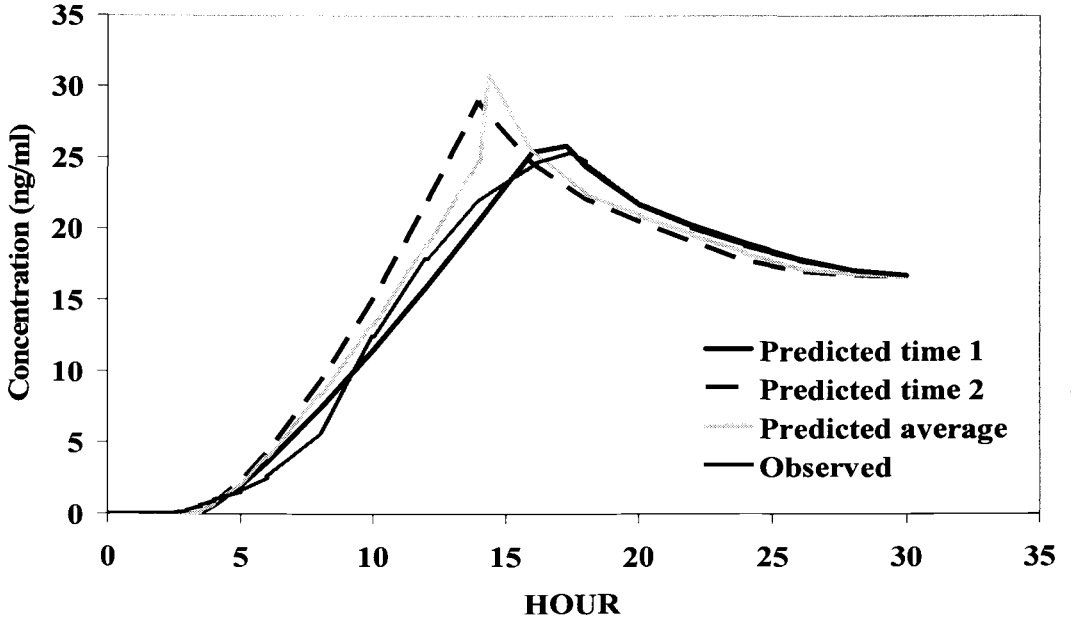


**Figure A.44: Convolution of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 15% coating.** Similarity factor 2 between observed and predicted average is 69.22993. Similarity factor 2 between predicted time 1 and time 2 is 49.25651.

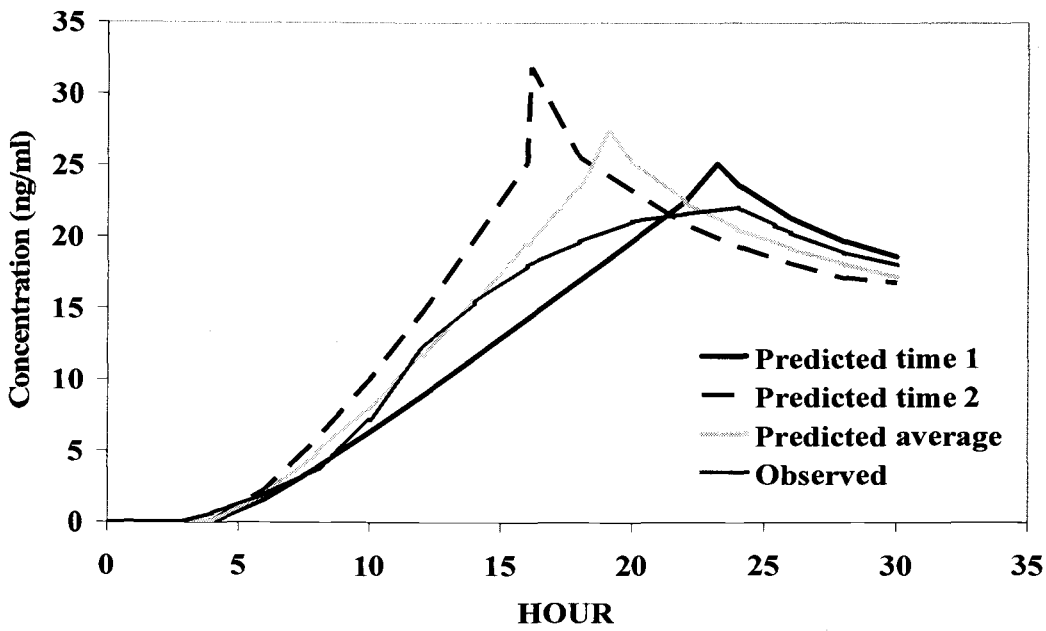


**Figure A.45: Convolution of cyclobenzaprine HCL from matrix tablet containing 30% HPMC-E5 and 18% coating.** Similarity factor 2 between observed and predicted average is 78.45837. Similarity factor 2 between predicted time 1 and time 2 is 43.07174.

Since the difference in predicted drug release profiles between tablets prepared at time 1 and at time 2 is small (< 20%), the variable coefficient of these two regression models (model with the fixed effect of time 1 and time 2) were averaged to obtain the new regression model which accounted for the effect of time. The new mathematical model equations for predicting n-value, lag time, and percent drug release at 6 and 10 hours are shown in equation 8-11.

N-value = $0.56125 + 0.01101\ hpmc + 0.09333\ coat - 0.00330\ coat^2$ equation (8)

Lag time = $-1.14225 + 0.1018\ hpmc + 0.1353\ coat + 0.002480\ (hpmc \times coat) - 0.0021\ hpmc^2$ equation (9)

DR6hr = $90.0855 - 0.7380\ hpmc - 6.3939\ coat + 0.1565\ coat^2$ equation (10)

DR10hr = $91.77165 - 0.7586\ hpmc - 0.1399\ coat^2$ equation (11)