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DEVELOPMENT OF ALGINATE-BASED MICROSPHERES FOR LUNG DELIVERY IN CYSTIC FIBROSIS

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SUMMARY

Cystic Fibrosis affects about one out of every 3,000 newborns and about one in 25 people are carriers, including in Africans and Asians. Cystic Fibrosis is a genetic disorder that affects mostly the lungs. Lung infections are treated with antibiotics which may be given intravenously, inhaled, or by mouth. Sometimes the antibiotic is used long term. Lung transplantation may be an option if lung function continues to worsen. Lung problems are responsible for death in 80% of people with cystic fibrosis.

Inhalation treatment using antibiotic is an alternative for lung delivery especially for cystic fibrosis, however, the therapeutic efficacy of inhaled drugs is limited by their rapid clearance in the lungs. Sustained release system in the lungs can improve therapeutic outcomes of inhaled drugs because they can retain the drug load within the lungs and progressively release the drug locally at therapeutic levels. This study presents the formulation strategies developed to control drug release in the lungs using microspheres system.

Microspheres are carrier for sustained drug release in the lungs which offer significant potential for prolonged drug release in the lungs. The microspheres composition can be adjusted to modulate drug release and can encapsulate compounds with high drug loading.

Pulmonary route is commonly used and has been well accepted as a portal for non-invasive drug delivery for many lung diseases and it is explored for decades as an alternative for systemic as well as local drug delivery. The pharmaceutical scientists are taking advantage of large peripheral surface area of lung for absorption and thinner alveolar epithelium providing shorter distance of air-blood exchange passage.

Alginate-based microspheres provide sustained release properties with several advantages such as minimum usage of toxic organic solvents and reduced reticuloendothelial system uptake due to stealth nature of alginate. The present study explores the in vitro benefits of antibiotic encapsulated alginate microspheres. The encapsulation method is by aerosolization technique considered as simple, easy, and produces small and uniform particle size.

The present study was to evaluate optimized a natural polymer-based inhalable drug delivery system using sodium alginate and antibiotic ciprofloxacin as a model. Small, good, smooth and spherical microspheres were found. In vitro release study showed sustained release profile was achieved; this is potential for pulmonary delivery. The antimicrobial potency against microorganism in lung disease was also effectively found. Stability of microspheres which stored at room temperature was found stable and able to maintain concentrations of antibiotic, however at high temperature, ciproflocain HCl-alginate microspheres were found stable for 21 days storage. Conducting long term stability test and in vivo study are highly recommended for further study.

FOREWORD

Thank God for the presence of Allah SWT over the blessings of mercy We can complete the final report of International Research Collaboration and Scientific Publication in 2018. This report is one of the conditions of obligation to complete the report of research. Preparation of this report can not be separated from the help of various parties, for that with a sense of humility I say thanks as much as possible to:

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CHAPTER 1
INTRODUCTION

Treatment of diseases in the lungs currently uses antibiotics to reduce infections and control inflammation (Gaspar et al., 2013). Inhaled treatment is increasingly being used for lung diseases. Inhaled antibiotics either singly or in combination are widely used for the treatment of cystic fibrosis (Noah et al, 2010). Fluroquinolone such as ciprofloxacin has good activity in gram negative aerobic bacteria (such as Escherichia coli) and gram-positive (such as Staphylococcus aureus) (Hardman et al., 2012). Pseudomonas aeruginosa has the highest susceptibility to ciprofloxacin, followed by amikacin, ceftazidine and ceftriaxone (Khalilzadeh et al, 2012). Ciprofloxacin is effective against respiratory pathogens such as Staphylococcus aureus and long-term treatment of cases of cystic fibrosis (Arnold et al., 2007).

The oral and intravenous form of ciprofloxacin has been used clinically to treat respiratory infections. However, intravenous or oral administration has a relatively unfavorable pharmacokinetic profile in the lower respiratory tract, including a short half-life of about 3-5 hours. Ciprofloxacin undergoes first past metabolism. Ciprofloxacin HCl has an oral bioavailability of about 70% and is classified into grade IV BCS due to low solubility and low permeability (Katzung et al., 2010; Olivera et al., 2011).

Delivery system through the lungs is one alternative delivery instead of other routes. Its bioavailability is high and does not have the first pass metabolism in the liver therefore it can deliver the drug well. The drug is readily absorbed and enters the systemic circulation because of the thin barrier and high vascularization that cover the lungs (Mukta and Christel, 2014). The pharmacological benefits of lung administration include systemic low exposure, reduced side effects, appropriate doses delivered to specific targets and no need additional doses (Courier et al., 2002).

Drug delivery for lung treatment in the form of microspheres offer an alternative to deliver high drug concentration directly to the site of action to improve the therapeutic effect and minimize the side effect (Geller, 2009; Traini, 2009). Active agent to be used as model for lung delivery in cystic fibrosis is antibiotic group with

microspheres delivery system as a promising approach for antibiotic inhalation. Infection which usually occurred in the lung delivery is cystic fibrosis.

Cystic fibrosis (CF) is a genetic disorder that affects mostly the lungs but also the pancreas, liver, kidneys, and intestine (Sanders etal, 2000). CF is most common among people of Northern European ancestry and affects about one out of every 3,000 newborns. About one in 25 people are carriers. It is least common in Africans and Asians (Sanders etal, 2000).

Long-term issues include difficulty breathing and coughing up mucus as a result of frequent lung infections (Smiths, 2002). Other signs and symptoms include sinus infections, poor growth, fatty stool, clubbing of the fingers and toes, and infertility in males, among others. Different people may have different degrees of symptoms. Lung infections are treated with antibiotics which may be given intravenously, inhaled, or by mouth. Sometimes the antibiotic azithromycin is used long term. Lung transplantation may be an option if lung function continues to worsen.

To enhance drug efficacy and reduce the dose and side effects, microspheres are alternative of lung delivery system, particularly in cystic fibrosis. Chemical properties of forming polymer may provide potential efficacy for antibiotic drug. The microspheres composition can be adjusted to modulate drug release and can encapsulate compounds with high drug loading.

Alginate-based microspheres provide sustained release properties with several advantages such as minimum usage of toxic organic solvents and reduced reticuloendothelial system uptake due to stealth nature of alginate. The present study explores the therapeutic benefits of antibiotic encapsulated alginate microspheres when administered by pulmonary route animal model as well as in vitro physical evaluation. The encapsulation method is by aerosolization technique considered as simple, easy, and produces small and uniform particle size.

The microspheres are spherical monolithic or agent therapeutic distributed in the matrix either as a dispersion of molecular or particle, or can be defined as a structure consisting of a continuous phase of one or more soluble polymer dispersed at the molecular level or macroscopic (Mathew et al, 2008). Microspheres have a particle size of 1-1000 nm (Karmakar, 2009). In the field of pharmaceuticals, drug delivery systems with technology microspheres used for the preparation of slow release and controlled,

reduce and even eliminate the irritation of the gastrointestinal tract, protect the drug from the ravages and support the spread of drugs distributed in the gastrointestinal tract resulting in absorption of the drug is more reproducible (Prasanth et al , 2011). The smaller the drug particle size, the greater the absorption in the gastrointestinal tract (Yoshie et al, 1996).

Ionotropic gelation technique is a method of preparation of microspheres by adding the drug solution into the polymer solution and the solution of the crosslinking agent, and then the process gellification for 24 hours (Prasanth, 2011). The advantage of using ionotropic gelation method in the preparation of the microspheres is able to maintain drug integrity so that the drug can be encapsulated without the use of organic solvents or elevated temperatures; this causes the drug to remain stable. In addition, the gelation ionotropic method was quite simple, fast, and cost-effective (Yeo et al, 2001). There are several factors that affect the manufacture of microspheres by the method of gelation ionotropic include a comparison of the ratio of drug-polymer, effect of concentration of crosslinker and polymer on the entrapment efficiency, size and distribution of particles, as well as the release profile of the drug (Manjanna, 2010).

An antibiotic activity test can generally be done by using two types of methods: dilution method and diffusion method. The diffusion method is more quantitative than other methods (Jorgensen and Ferraro, 2009). To understand the activity of microspheres against bacteria measurement of inhibitory by diffusion method of hole or well because this method influence of particulate material can be finalized compared to dilution method, need of sample in small amount, work procedure which relative easy and cheap, besides easy data result interpreted (Hadacek and Greger, 2000; Jorgensen and Ferraro, 2009).

Staphylococcus aureus and Escherichia coli are widely used in the study of antibiotic activity of ciprofloxacin (Mughal et al., 2009). Potential tests were performed by comparing the inhibition of ciprofloxacin of the microsphere formula with standard ciprofloxacin against the test bacteria. In this method, a 3: 3 design is used, where both standard and sample are diluted to 3 concentrations (MOH, 1979). Test the potential of ciprofloxacin HCl to bacteria using nutrient agar media. The inhibitory ability of ciprofloxacin HCl is measured based on the inhibitory zone diameter (Verma and Singh, 2015).

Preparations of alginate-based microspheres for lung delivery in terms of evaluation of physical characteristics include shape, particle size, surface appearance, content of drug, the water content of the microspheres. Based on the above, this study was to develop and evaluate a natural polymer-based inhalable drug delivery system using sodium alginate and antibiotic as a model for cystic fibrosis treatment. Antimicrobial activity of ciprofloxacin HCl-alginate microspheres to Escherichia coli and Staphylococcus aureus will be determined.

1.1. Research Problems

1. How is the optimum physical characteristic of ciprofloxacin-loaded alginate microspheres produced by aerosolization for lung delivery?
2. How is the physical stability of ciprofloxacin-loaded alginate microspheres in several intervals of storage time?
3. How is the antimicrobial activity of ciprofloxacin-loaded in alginate microspheres for lung delivery?

CHAPTER II
LITERATURE REVIEW

2.1. Antibiotic for cystic fibrosis

The most important bacterial pathogen associated with cystic fibrosis from the perspective of prevalence and pathogenicity is Pseudomonas aeruginosa (Geller, 2009). P. aeruginosa is found in almost 80% of patients with cystic fibrosis by 18 years of age; overall 54.4% of the patients are infected. Once P.aeruginosa has established a home in the respiratory tract of cystic fibrosis patients, the clinical course can worsen. Infection with chronic, mucoid P.aeruginosa is associated with poor growth, more rapid decline in lung function, increased need for antibiotic treatment and hospitalization, and earlier death. Therefore, effective antimicrobial therapies targeting this pathogen are central to the management of cystic fibrosis.

Antibiotics used to treat P. aeruginosa include amino-glycosides, βlactams, polymyxins, and fluoroquinolones. Delivering antibiotics via aerosol makes particularly good sense for CF, since the infection is mostly limited to the endobronchial space.

Antibiotics can be categorized by the pharmacodynamic (PD) parameters that best predict efficacy. In the case of pseudomonas, most βlactam antibiotics (synthetic penicillins, cephalosporins, monobactams, and carbapenems) display "time-dependent" killing (ie, the duration of time the drug concentration remains above the MIC of the bacteria). Conversely, aminoglycosides and fluoroquinolones display a "concentration-dependent" pattern (ie, the ratio of maximum drug concentration [or the area under the concentration-time curve] to the MIC (Ambrose et al, 2007). These agents (but not most lactams) also demonstrate a post antibiotic effect with P. aeruginosa, which is a time period of bacterial growth suppression after the drug concentration falls below the MIC. Thus, β lactams may require more frequent doses to keep the lung concentration above the MIC, whereas aminoglycosides and fluoroquinolones use "shock-and-awe" concentrations to kill the microbes, and are effective with less frequent dosing (Ambrose et al, 2007).

2.2. Lung Delivery

The respiratory tract is an important for lung delivery. The respiratory tract starts from the nose and ends deep inside the lungs in the alveolar sac. To understand the anatomical and physiological structures of the respiratory tract as one of the drug delivery routes, the respiratory tracts are grouped into sections (Glyn Taylor and Ian Kellaway, 2001):

1. Nasopharyngeal region (NP) The nasopharyngeal region is also called the upper respiratory tract, consisting of the nose, throat, pharynx, and larynx.
2. Tracheo-bronchial region (TB) The trakheo-bronchial region is also called the middle respiratory tract, starting from the larynx continuously extending over the trachea, bronchi, and ending in the bronchioles.
3. Alveolar region (A) The alveolus area is also referred to as the inner respiratory tract (peripheral) or also known as the pulmonary region. It consists of bronchioles, alveolar ducts, and alveoli.

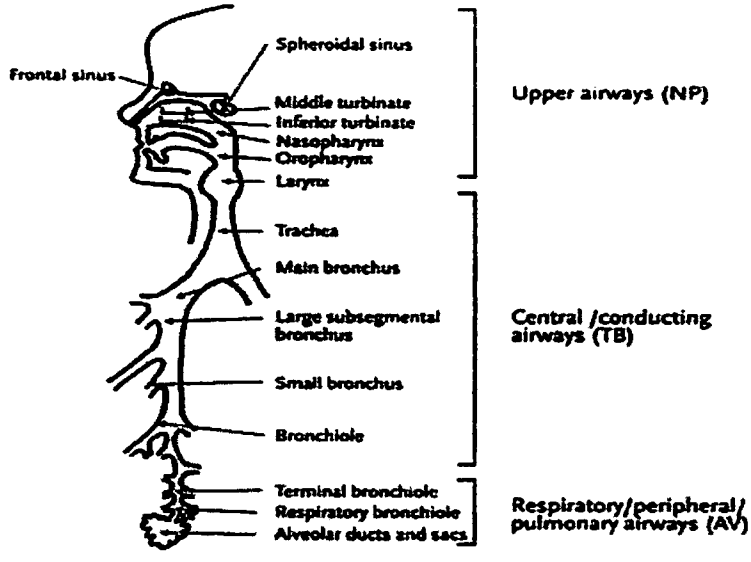


Figure 2.1. Respiratory tract organ of human (Glyn Taylor and Ian Kellaway,2001).

Delivery of drugs through the lungs refers to the approach, formulation, technology, and system to transport the drug compounds in the body necessary to achieve the desired therapeutic effect safely to the lungs. Delivery of drugs through the lungs is a potential route for delivering drugs locally as well as systemically (Milala, 2013).

Particles of more than 10 μm size will collide on the upper respiratory tract and are easily excreted by coughing, swallowing and mucosiliary clearance processes. Particles of 0.5-5 μm size can avoid collisions occurring in the upper respiratory tract and will be deposited through collisions and sediments in the trachehebronchial and alveolar areas. If the particle size is between 3-5 μm it will be fully deposited in the trachehebronchial region and if the size is less than 3 μm then is likely to be deeper in the alveolar region (Glyn Taylor and Ian Kellaway, 2001). Dry powder for inhalation is formulated in the form of loose agglomerates of microporized drug particles with an aerodynamic particle size of less than 5 μm, or in the form of an interactive mixture with micronized drug particles attached to a larger-sized carrier surface. Drug delivery for respiratory tracts with particles of 2-5 μm yields optimal benefits, whereas to produce a systemic effect, it takes particles less than 2 μm in size. Inhalation is the process of treatment by inhaling the drug to get directly into the lungs as the target organ. Meanwhile, nebulisation is a way to convert a solution or suspension of a drug into a vapor to be inhaled through the nose by breathing normally (Milala, 2013)

Drug formulations provide an important role in the production of effective inhalation. Treatment with inhalation is not only important in terms of pharmacology of the active substance but also must be delivered efficiently, appropriately targeted and left lunged until the desired pharmacological effect occurs. Formulation is done to make the drug lag in the lungs in accordance with the desired time and avoid the mechanism of cleaning the lungs. The formulation of dry powder for inhalation involves micronization using a variety of excipients such as lipids, polymers or carrier systems such as lactose. One formulation of dry powder for inhalation products is to use biodegradable polymers. Biodegradable polymer microspheres have been studied as carriers in slow drug delivery through the lungs. Mucoadhesive polymers can prolong pharmacokinetic drugs by avoiding mucous clearance (Labiris and Dolovivich, 2003).

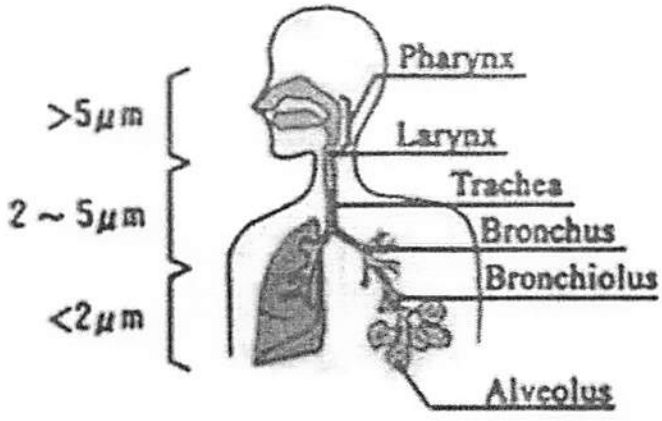


Figure 2.2. The particle size determines the particle deposition in the lungs (El-Sherbiny et al., 2015).

2.3. Microspheres

Microspheres are free-flowing powder, containing a biodegradable polymer and ideally have a particle size of less than 200 μm (Alagusundaram et al., 2009). Microspheres having a small size (microns) so that it can easily penetrate various types of capillary having a micron size. Microencapsulation for use as a sustained release oral use and to reduce or eliminate the irritation of the gastrointestinal tract. In addition, microparticle delivery systems more uniform spread of the gastrointestinal tract (Prasanth et al., 2011).

2.3.1. Preparation of microspheres

Preparation of microspheres must meet the following criteria:

- Have a high ability to trap drug
- Stable during preparation and after synthesized with a shelf life that is clinically acceptable
- The size of particles is limited and can be dispersed on the carrier, especially for injection
- Can release the drug substance in the long term
- Biocompatible and biodegradable (Alagusundaram et al., 2009).

Different methods of making microspheres depend on particle size, route of administration and duration of drug release (Prasanth et al., 2011). Preparation of

microspheres can be used in several ways, such as Spray Drying, Solvent Evaporation and Extraction, Supercritical Fluid, and Ionotrophic gelation. Alginate is capable of forming a gel in the presence of alginate ion bond either monovalent, divalent, or polyvalent cations (Penman et al, 1972). In some other references, this technique is also called the nebulizing techniques. In this method, the internal diameter of the spray holes having a size of about 0.5 μm-1μm. High-pressure air then flows to the material that will be used as a core material in a container (Benoit et al, 2000). Then it mixed with a solution containing ions crosslinking agent using monovalent, divalent, or polyvalent cations while stirring. Microspheres are desired to be formed after mixing with the speed and time.

2.4. Alginate

Alginates are anionic polysaccharides and hydrophilic nature. Alginate is one ingredient that is abundant, is derived from brown seaweed and bacteria. Alginate is composed of monomers (1-4) β-D-mannuronic acid (M) and α-L-guluronic acid (G). The sections are composed of three different polymers that form residue sequence D, M and residue residue sequence that alternately MG (Jinchen and Huaping, 2013).

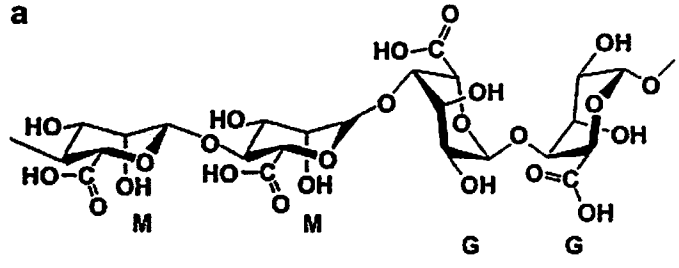


Figure 2.3. Alginate chemical structure

Here is a physico-chemical property of alginate:

Acidity: pH 1.5-3 for dispersion in solution with concentrations of 3% (w / v).

Density: 1,601 g / cm3

Humidity: 7.01%

Solubility: Soluble in alkali hydroxide, to form a solution viscous, very slightly soluble or practically insoluble in ethanol and other organic solvents. Alginate is not soluble in

water, but expands and is able to absorb the water for 200-300 times the weight of water.

Viscosity: viscosity increases with increasing concentration, 0.5% (w / w) dispersion in solution has a viscosity of 20 mPas, while 2.0% (w / w) dispersion in solution viscosity is reached 2000mPas. Viscosity decreases with increasing temperature. An increase of 1 °C would lower the viscosity of 2.5%. At low concentrations, the viscosity of the alginate can be increased by the addition of calcium salts such as calcium citrate.

Stability: Alginate will terhidrolisasi at warm temperatures,forming molecular weight materials and lower viscosity. Incompatibility: Incompatible with strong oxidizing agents, alginic acid forming insoluble salts with alkaline earth metals and group III metals except magnesium (Handbook of Pharmaceutical Exipients, 2009).

Alginate has an important role in maintaining the stability of biomaterials using alginate. The molecular weight of the alginate affects the rate of degradation and biomaterial characteristics of the use of alginates. The larger the molecular weight, the smaller the degradation reactions of hydrolysis, thus causing the release of drugs more slowly. FDA approves alginate as a biomaterial which is important for a variety of uses in medicine, nutritional supplements and others. Alginate can form a gel and solid microspheres as drug delivery systems, typically made by the reaction of a cross connect and suitable for the encapsulation of cells, growth factors and protein drugs (Jinchen and Huaping, 2013).

2.5. Crosslinking Agent

The most common method used in making alginate hydrogels is to add divalent cations, the crosslinking agent. The presence of divalent cations can form a gel when divalent cations interact with section G of the bridge ionic monomer.

At the alginate solution, part of monomer M form weak bonds with divalent cations. However, interactions between monomers G and divalent cations to form closer ties. Crosslinking agent commonly used in alginate include Ca2+, Mg2+, Fe2+, Ba2 + or Sr2+. Ca2+ is one of the best options as agents continued crosslink in alginate (Jinchen and Huaping, 2013). Ca2+ binding over poliguluronat acid group (G) of alginate in the form of two-dimensional planar, yields a so-called egg-box (Gulati et al., 2011).

In past studies, the formula with continued crosslinker Ca2+ resulted in higher antibody titers than formula with continued crosslinker BaCl2. This occurs because of differences in affinity between the microspheres with continued crosslinker CaCl2 and BaCl2 (Hariyadi et al, 2015).

2.6. Microorganism in lung disease

2.6.1. Staphylococcus aureus

Classification of Staphylococcus aureus

Division: Protophyta

Class: Schizomycetes

Species: Staphylococcus aureus (Holti et al., 1994)

Stapylococcus is a gram-positive or oval gram-positive bacterium usually arranged in the form of an irregular cluster like grapes. It grows well in various bacteriological media in aerobic atmosphere and at optimum pH of 7.4. The temperature limits for growth are 15-40 °C, while the optimum growth is 37 °C. Colonies on a dense medium are round, soft and shiny. Staphylococcus aureus usually forms a gray colony to golden yellow. Staphylococcus is relatively resistant to drying (50 °C for 30 minutes), salt resistant and grows well on medium containing 9% NaCl, but is easily inhibited by substances certain chemicals, such as 3% hexachlorophen. Stapylococcus is a normal flora of human skin, respiratory and gastrointestinal tract. People suffered with Staphylococcus aureus in the nasal is as much as 40-50% of the population. Staphylococcus aureus is the main pathogen in humans (Jawetz et al., 2005).

2.6.2. Escherichia coli

Classification of Escherichia coli

Division: Proterobacteria

Class: Gamma Proteobacteria

Species: Escherichia coli (Holti et al., 1994)

Escherichia coli is a gram-negative, short-stem, has a flagellum, measuring 0.4-0.7 μm and has a hoop. Escherichia coli grow well in almost all seed media, and can be lactose fermentation. The optimum temperature is 37 ° C. Escherichia coli is a normal flora found in the gut. These bacteria are also found in small amounts as part of the

normal flora of the respiratory and genital system. Bacteria become pathogens when they reach the normal intestinal outer tissue. Most places that have clinical infections are urinary tract, biliary system, abdominal cavity, prostate gland, lung, bone; meningen can become a disease site. Local infection may occur when immunity is decreased (Jawetz et al, 2005).

2.7. Evaluation of antimicrobial activity

Antimicrobial activity is measured to determine the potential of antimicrobial agents in the solution, its concentration in body fluids or tissues and the sensitivity of known drug-causing microorganisms. Determination of sensitivity of pathogenic bacteria to antimicrobials can be done with one of the two main methods of dilution or diffusion (Jawetz, et al., 2005).

1. Dilution method

This method uses antimicrobials with gradually decreasing levels either with liquid or solid media. The liquid dilution / broth dilution test method is used to measure minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). This method is carried out with a series of antimicrobial dilutions on media that have been added test microbes. The smallest test solution with the smallest visible content in the absence of microbial growth is defined as MIC. The prescribed solution was reprocessed on the test medium without bacterial or antimicrobial addition and incubated for 18-24 hours. Liquid media that remains clear after incubation is defined as MBC. The solid dilution method is similar to the liquid dilution method but uses a solid medium.

The advantage of this method is that one of the concentrations of the antimicrobial agents tested can be used to test several test microbes (Pratiwi, 2008). Sensitivity test dilution way to take time and its use is limited to certain circumstances. The sensitivity test of liquid dilution using reaction tubes is impractical and rarely used, but now there is a simpler, more widely used way, using a microdilution plate. The advantage of the liquid microdilution test is that it gives quantitative results indicating the amount of antimicrobial required to kill bacteria (Jawetz, et al.,2005).

2. Diffusion Method

This method is to determine the antimicrobial effect by measuring the diameter of the antimicrobial resistance zone against bacterial growth. These methods include paper disc method, fluid method in ring, and hole method. Disc diffusion method (Kirby test and odor) to determine antimicrobial activity by using a disc containing antimicrobial agent placed on agar medium. Clear areas indicate obstacles growth of microorganisms (Pratiwi, 2008). The use of single discs in each antibiotic with good standardization can determine whether bacteria are susceptible or resistant by comparing standard barrier zones for the same drug (Jawetz, et al., 2005). The wellbore or hole method is suitable for solution because by that method the influence of particulate material can be minimized than any other method. The specific advantage of this method is that it takes a small sample and can test more than six samples per cup for one type of microbe (Cole, 1994; Hadacek and Greger, 2000). The number and location of the hole is tailored to the destination research, then the hole is filled with the tested solution. After incubation, bacterial growth was observed to see whether or not there were obstruction areas around the hole (Kusmiyati, 2007).

CHAPTER III
RESEARCH AIMS AND BENEFITS

3.1. Research Aims

1. To select optimized formula of ciprofloxacin-alginate microspheres produced by aerosolization.

2. To evaluate the physical stability of ciprofloxacin-loaded alginate microspheres in several intervals of storage time.

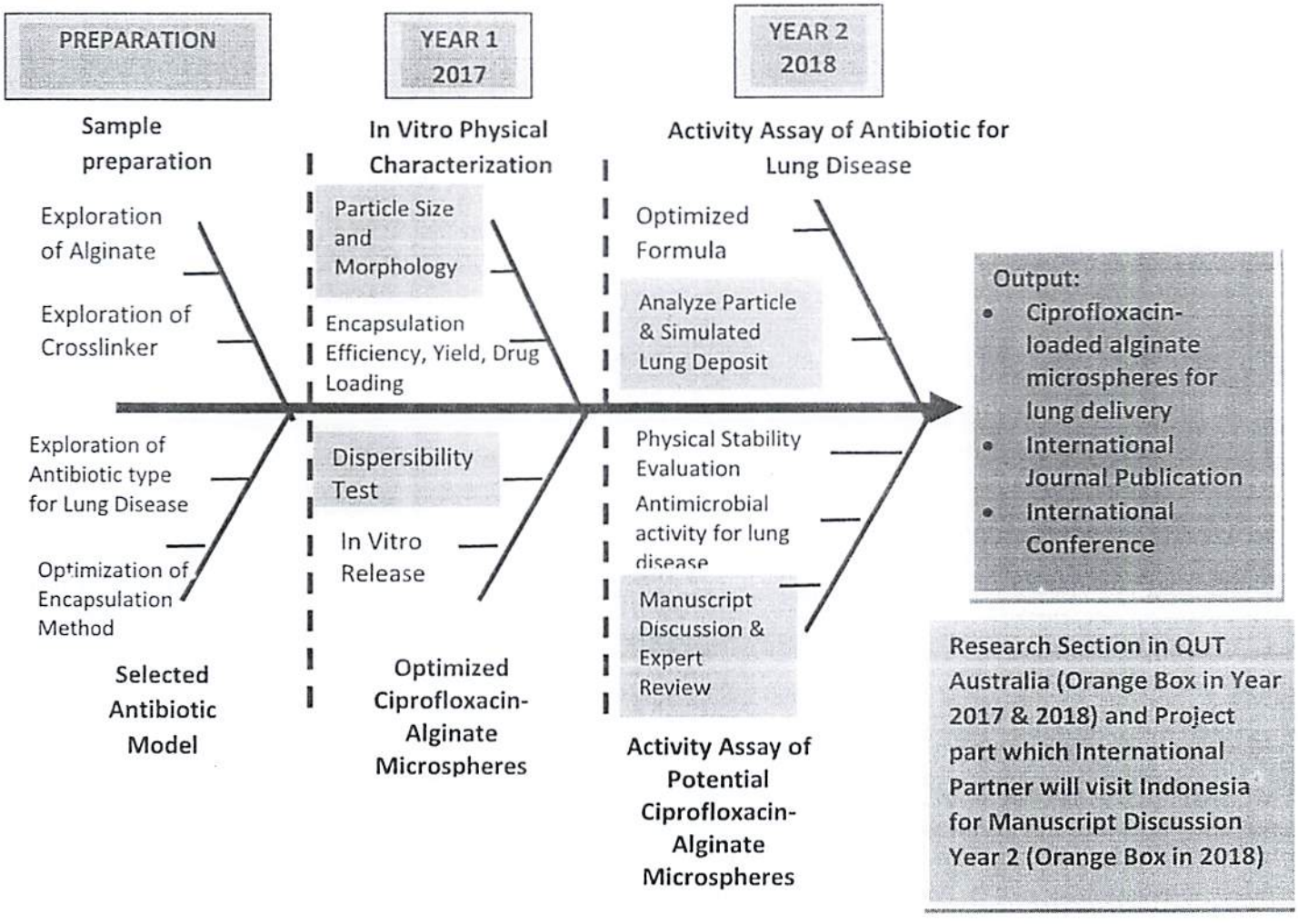
3. To determine the antimicrobial activity of ciprofloxacin-loaded in alginate microspheres for lung delivery.

3.2. Benefits

The benefits of this study is expected to develop system of alginate microspheres with a model drug using gelation ionotropic method with aerosolization techniques. A significant benefit and contribution to the study of science, especially in the field of development of pharmaceutical dosage antibiotic-alginate microspheres in the lung delivery particularly in cyctic fibrosis in Indonesia is targeted to contribute to the development and applied research in hospitals and the community.

CHAPTER IV
RESEARCH METHOD

Conceptual Framework of Multiyears Research



4.1. Materials and method

4.1.1. Materials

The materials used in this study is antibiotic aminoglycoside model Ciprofloxacin (Pharmaceutical grade); Sodium Alginate pharmaceutical grade (Wako Pure chemical Industry Ltd.); $CaCl_2.2H_2O$ pharmaceutical grade; Sodium Citrate pharmaceutical grade; Phosphate Buffer Saline (PBS), Distilled water (BRATACO) and Distilled water.

4.1.2. Instrument

The instruments include analytical balance (electronis Balance Chyo JP-160), optical microscopes (Axioskop 40 Zeiss), pH meter (Eutoch Instrument pH 700), centrifuges Rotofix-32 (Hettich Zentrifugen), Plate Stirer (Dragon Lab MS pro), Freeze Dryer (EYELA

FD-81), atomizer aerosolization (35μm orifice size and pressure of 40 psi), a glass tools, optical microscope (Olympus), UV-Vis spectrophotometer (Carry-50), Spectrophotometer FT IR (Perkin Elmer Instrument), Differential Thermal Apparatus (Mettler Toledo FP 900 FP 85 DTA), Scanning Electron Microscopy (SEM), Freeze dryer, Moisture Content Analyzer.

4.2. Preparation of antibiotic-loaded alginate microspheres

Preparation of antibiotic-loaded alginate microspheres made with ionotropic gelation method using aerosolization techniques, with concentration of antibiotic from 2.0 to 3.5%. Drug was dissolved in a solution of alginate polymer and uses a crosslinker $CaCl_2$ at concentration of 0.5-1.5 M. Each formula is crosslinked for 120 minutes and stirring is carried out at a speed of 1000 rpm. Formula antibiotic-alginate microspheres are washed by centrifugation and drying techniques using a freeze dryer at -80 ° C for 29 hours with the addition of 5% maltodextrin lyoprotectant as a stabilizer. Furthermore, antibiotic-loaded alginate microspheres that form will be evaluated.

Formula which will be used forphysical characterization of antibiotic-loaded alginate microspheres were shown in Table 4.1.

Tabel 4.1. Formula ofciprofloxacin HCl-loaded alginate microspheres

Formula / Material	F1	F2	F3	F4	F5	F6	F7	F8
Ciprofloxacin HCl	0,1%	0,1%	0,1%	0,1%	0,1%	0,1%	0,1%	0,1%
Alginate	2,0%	2,0%	2,5%	2,5%	3,0%	3,0%	3,5%	3,5%
$CaCl_2$	1,5M	0,5M	1,5M	0,5M	1,5M	0,5M	1,5M	0,5M
Maltodextrin	5%	5%	5%	5%	5%	5%	5%	5%

F1: Formula Antibiotic-loaded alginate microspheres with concentration of antibiotic 0,1%, alginate 2% and $CaCl_2$ 1,5M
F2: Formula Antibiotic-loaded alginate microspheres with concentration of antibiotic 0,1%, alginate 2% and $CaCl_2$ 0,5M
F3: Formula Antibiotic-loaded alginate microspheres with concentration of antibiotic 0,1%, alginate 2,5% and $CaCl_2$ 1,5M
F4: Formula Antibiotic-loaded alginate microspheres with concentration of antibiotic 0,1%, alginate 2,5% and $CaCl_2$ 0,5M
F5: Formula Antibiotic-loaded alginate microspheres with concentration of antibiotic 0,1%, alginate 3% and $CaCl_2$ 1,5M
F6: Formula Antibiotic-loaded alginate microspheres with concentration of antibiotic 0,1%, alginate 3% and $CaCl_2$ 0,5M
F7: Formula Antibiotic-loaded alginate microspheres with concentration of antibiotic 0,1%, alginate 3,5% and $CaCl_2$ 1,5M

F8: Formula Antibiotic-loaded alginate microspheres with concentration of antibiotic 0,1%, alginate 3,5% and $CaCl_2$ 0,5M

4.3. Characterization of antibiotic-loaded alginate microspheres

4.3.1. Identification of melting point using DTA FP 900 *Thermal System*

The material was weighed 3-5 mg, and then inserted into the sample pan and closed. Sample pan was inserted into the sample holder. Sample pan used aluminum crucible with a maximum temperature of 350 °C. Heating program is run with a heating rate of 5 °C/min, when equilibrium is obtained after the initial melt temperature is reached. The test results obtained were compared with the literature (MOH, 1995).

4.3.2. Infra red spectrum identification using KBr pellet technique

1 mg of substance was crushed with KBr powder dry, then compressed with a hydraulic press equipped puller water vapor in order to obtain a thin translucent plates. Infrared spectra obtained were compared with the infrared spectra of the library.

4.3.3. Particle size distribution

This study was performed by using an optical microscope.

1. The scale of ocular was calibrated as follows:

a. Ocular micrometer and objective position is in place.

b. Both scales were observed to be seen clearly under the microscope.

c. The initial line of ocular scale with the start line is inline with objective scale, then determined the appropriate line on both scales coincides.

d. Ocular scale is determined, for example ocular scale 9 = 10 objective scale, then one scale ocular = 10/9 objective scale.

2. The microspheres to be observed is placed on an object glass.

3. The objective micrometer taken, replaced with a glass object that contains the sample, and then starts the measurement of the particle diameter.

4. Groupings from the smallest particle size is determined and the greatest of all samples, divided into several intervals and class.

5. Average diameter and of particle size distribution curve was determined.

4.4. Drug loading

Procedure to measure drug loading is as follows:

1. 10 mg samples of microspheres were prepared.

2. 5 mL of sodium citrate pH 6.0 was added in the sample microspheres and was stirred for 24 hours at a speed of 1000 rpm.
3. The resulting clear solution had an absorbance was measured by UV-Vis spectrophotometer at the maximum wavelength of antibiotic.

4.5. Morphology and shape evaluation

To evaluate the shape and surface of the microspheres was done using optical microscopy and photos are taken using the camera. Moreover, it can also be observed using Scanning Electron Microscopy (SEM). Examination by SEM provided higher resolution compared to optical microscopy.

4.6. Swelling Index

Swelling index determination was done by weighing 100 mg of microspheres and then adding 5 ml of PBS pH 7.4 in a vial. This swelling index determination was carried out for 24 and 30 hours. After the observation time is reached, the wet microspheres are filtered using filter paper. After there was no PBS dripping from the filter paper, the wet microspheres are transferred to the dry filter then the microsphere was turned back and forth to remove adsorbed water on the surface of the microspheres until the filter paper was not too wet. After that the wet microspheres are ovened at 37 ° C for 2 hours or until the weight of wet microspheres was constant. After drying, the microspheres are weighed as heavy swelling.

Swelling index observations were also carried out based on the size of the microspheres. Wet microspheres were taken slightly to observe the size of the microspheres when swelling with an optical microscope.

4.7. Moisture Content Test

Moisture Content of the microspheres were analysed using Moisture Content Analyzer after drying process using Freeze Dryer.

4.8. In Vitro Release Study

100 mg microspheres were added into the erlenmeyer containing 100 ml of HCl pH 1.2 and was stirred at 37 ± 0.5 ° C 100 rpm. Sampling medium of a 5 ml at regular intervals and medium was replaced with same volume of new medium solution, taking snippets of samples was measured absorbance at the maximum wavelength of drug. The same test was also

conducted in the medium of PBS pH 7.4. Number of drug release was calculated at interval time.

4.9. Antimicrobial Activity Test

4.9.1. Sterilization of tools and media

All equipment needed to work with bacteria should be sterilized including petri dishes, cooking and blue tip for micropipets. In addition to equipment is also done sterilization of the media to grow bacteria. Sterilization of tools and media is carried out in an autoclave at a temperature of 121°C for 20 minutes. Sterile media and tools are then stored in a room with a temperature of 25 ± 1 °C before use.

4.9.2. Preparation of Media

The nutrient media to be prepared by weighing 28 grams of powder is suspended into Erlenmeyer with distilled water to 1000 ml, then the media is heated briefly while occasionally stirring until boiling and forming clear solution. The nutrient solution is added into 10.0 ml as the base layer and 8.0 ml as the seed layer. Then it is sterilized by autoclaving at 121 °C for 15 minutes.

4.9.3. Preparation of 0.9% NaCl solution

NaCl is weighed as much as 0.9 g, then it is dissolved with aquadest until completely dissolved, the distilled water is added until reach the mark and sterilized with autoclave at a temperature of 121°C for 15 minutes.

4.9.4. Preparation of bacterial inoculum

The cultures of Pseudomonas aeruginosa and Staphylococcus aureus ATCC 25923 are grown on the surface of the nutrient medium so that it is evenly incubated at 37 ° C for 24 hours. From the stock of bacterial cultures that have grown, it is taken with a sterile ose needle and then suspended into a tube containing 5 ml of 0.9% NaCl solution. Keep shaking until all the colonies on the surface are removed and suspended in a 0.9% NaCl solution. The inoculum is then measured transmittance at a wavelength of 580 nm with a UV-Vis spectrophotometer. The suspension transmittance is adjusted to 25%, if the transmittance does not reach 25% then the suspension is diluted by adding a sterile solution of 0.9% NaCl, if more than 25% transmittance, the bacterial colonies is added. The blank is sodium chloride solution.

4.9.5. Preparation of phosphate buffer solution

a. Stock Solution A: 0.1 M of Dibasic Sodium Phosphate or Na2HPO4 solution (17.799 g Na2HPO4.2 2 H 2 O is dissolved in 1 L Aquadest)

b. Stock Solution B: 0.1 M solution of Monobasic Sodium Phosphate or NaH2PO4 (15.601 grams NaH2PO4.2H2O is dissolved in 1 L Aquadest)

Preparation of phosphate at pH 7,40 + 0,05 by mixed 80,80 ml stock solution in 19,20 ml of stock solution B.

4.9.6. Optimization of minimum inhibitory concentrations and dilution

The minimum inhibitory concentration (MIC) of Ciprofloxacin against Staphylococcus aureus bacteria is 0.25 µg/ml - 2 µg/ml (Andrews, 2011). Therefore, standard solution of ciprofloxacin with 0.06 µg/ml - 2 µg/ml was prepared. For the MIC of ciprofloxacin against the gram-negative bacteria Escherichia coli is 0.004 µg/ml-2 µg/ml (Kepel et al, 2015). Therefore, standard solution of ciprofloxacin with 0.06 µg/ml -2 µg/ml was prepared. Nutrients for containing 10.0 µL of bacteria! inoculum are included in the petri dish. Several series of standard solutions of ciprofloxacin are introduced in the amount of 50.0 µL into the hole formed in petri dishes. Incubate at 37 °C for 24 hours. Observe the presence or absence of a drag zone. The lowest level inhibited zone is the minimum inhibitory content of ciprofloxacin.

Dilution optimization is carried out to avoid overlapping drag zone in petri dish. This optimization is based on the highest levels of the sample. The highest standard of ciprofloxacin (S1) solution is created according to the highest sample level (U1). The dilution of 2 levels with a certain ratio in both sample and standard solution (U2, S2, S3, and U3) were performed and a petri dish that contains 10.0 µL of nutrient agar and bacteria was prepared. The solution into the hole in the petri dish is put followed by incubationat 37 °C for 24 hours.

4.9.7. Standard solution of ciprofloxacin

Ciprofloxacin HCl equivalent of ciprofloxacin 50 mg is weighed and dissolve in phosphate buffer pH 7,40 + 0,05 about 500,0 ml. Standard parent solution with concentration 100 ppm was achieved.

The liquefied base layer (45-50 °C) is poured into a 9 cm dish, allowing to solidify. A bacterial inoculum of 10.0 µL is inserted into a liquid seed layer containing 8 ml of nutrient agar that has cooled to 45-50 °C, then divortex to homogenize the bacteria. The seed layer is poured into a petri dish that already contains the base layer, let it solidify. After the media solidifies, create a hole on the surface of the media for as many as 6 holes. Standard solutions and samples were put into holes with respective volumes of 50.0 µL and incubate at 37 °C for 24 hours. The zone diameter is measured by measuring the diameter of the clear area. Replication 3 times for each sample was tested.

4.10. Physical stability of ciprofloxacin HCl-alginate microspheres

Ciprofloxacin-alginate microspheres are stored at two different temperature conditions, 25 ± 2 °C and 50± 2 °C for 30 days of storage periods with observation intervals of 0, 7, and 30 days. Observations of its stability in the form of measurement of ciprofloxacin levels in alginate microspheres were observed for any changes during the observation.

The 6.5 gram dry ciprofloxacin-alginate microspheres were inserted into a brownish yellow glass vial and sealed and then stored at 25 ± 2 °C and 50 ± 2 °C for 30 days of storage period, with observation time interval of 0, 7 and 30 days. The observed changes were including organoleptic, shapes and surfaces, drug loading, entrapment efficiency, and moisture content of dry microspheres observed until day 30 storage.

4.11. Data Analysis

Data parameter calculation results were analyzed by using statistical method of one-way ANOVA using SPSS 20 for windows Evaluation version with a degree of confidence of 95% ($\alpha = 0.05$). While parametric test using t-test was done to see the significance of difference of potential value of antibiotic at $\alpha = 0,05$, the difference was significant if obtained result $p < 0,05$.

4.12. Research Outcome

Research Outcomes of the 2nd year include:

1. Scientific publication in scientific International Journals
2. The application of science and technology in the field of pharmacy

CHAPTER V
RESULTS AND OUTPUT

5.1. Drug Loading and Entrapment Efficiency of Ciprofloxacin -Alginate Microspheres

The result of Drug Loading andEntrapment Efficiency of ciprofloxacin-alginate microspheres is shown in Table 5.1.

Table5.1. Results of Physical Characterization of Ciprofloxacin HCl-Alginate Microspheres

Formula	Physical Characteristics	
	Drug Loading (%) ± SD	Entrapment Efficiency (%)± SD
F1	15.37	14.81
F2	26.08	22.45
F3	22.50	23.39
F4	49.46	56.43
F5	24.61	25.51
F6	72.80	84.40
F7	75.20	95.40
F8	79.61	95.29

Physical Characteristics Microspheres which have highest drug loading and entrapment efficiency, which is Formula F7 and F8 were then selected to characterize physically in terms of FTIR spectrophotometry, DTA, particle size, MC and morphology.

5.2. FTIR Spectrophotometre of Optimized Microspheres

FTIR spectrophotometer of Formula 7 and 8 were characterized as shown in table 5.2 and 5.3.

Table 5.2.FTIR spectrophotometer of Formula 7

Chemical Compound	Wavelength Number (cm^{-1}) Microspheres	Wavelength number according to references		
		Ciprofloxacin HCl	Natrium Alginate	Maltodextrin
Amina Secondary	3673,48 cm^{-1}	Peak at 3700 - 3000 cm^{-1}		
C=O *stretching*	1733,64 cm^{-1} 1729,64 cm^{-1}	1750-1700 cm^{-1}		
OH and H-Intermolecular	3431,40 cm^{-1}	3530,23-3373,32 cm^{-1}		

Bond				
Quinolon	1648,58 cm^{-1}	1650-1600 cm^{-1}		
OH *stretching*	3431,40 cm^{-1} 3419,40 cm^{-1} 3416,40 cm^{-1}		3500 - 3200 cm^{-1}	3400 cm^{-1}
C-H *stretching*	2974,54 cm^{-1}		3000 - 2280 cm^{-1}	
Carboxylate acid	1424,68cm^{-1}		Asimetri : 1608 cm^{-1} Simetri : 1423 cm^{-1}	
C-O *stretching*	1340,65 cm^{-1}		1350 - 1027 cm^{-1}	1200 - 980 cm^{-1}
Guluronate fingerprint	1023,66 cm^{-1}		947, 64 cm^{-1}	

Table 5.3FTIR spectrophotometer of Formula 8

Chemical Compound	**Wavelength Number (cm^{-1}) Microspheres**	**Wavelength number according to references**		
		Ciprofloxacin HCl	**Natrium Alginate**	**Maltodextrin**
Amina Secondary	3659,73 cm^{-1}	Peak at 3700 - 3000 cm^{-1}		
C=O *stretching*	1739,81 cm^{-1} 1732,81 cm^{-1}	1750-1700 cm^{-1}		
OH and H Intermolecular Bond	3449,66 cm^{-1} 3427,66 cm^{-1}	3530,23-3373,32 cm^{-1}		
Quinolon	1649,77 cm^{-1} 1637,77 cm^{-1}	1650-1600 cm^{-1}		
OH *stretching*	3467,50 cm^{-1}		3500 - 3200 cm^{-1}	3400 cm^{-1}
C-H *stretching*	2999,76 cm^{-1} 2925,73 cm^{-1}		3000 - 2280 cm^{-1}	
Carboxilic acid	1424,66 cm^{-1}		Asimetri : 1608 cm^{-1} Simetri : 1423 cm^{-1}	
C-O *stretching*	1331,79 cm^{-1} 1282,76 cm^{-1}		1350 - 1027 cm^{-1}	1200 - 980 cm^{-1}
Guluronate fingerprint	1020,63 cm^{-1}		947, 64 cm^{-1}	

Table 5.1 and 5.2explained that the microspheres formulation process was able to interact between alginate and $CaCl_2$ to produce microspheresentrappingCiprofloxacin HCl. This was

shown from the groups of ciprofloxacin HCl, namely the secondary amine group, C = O stretching, OH and the intermolecular bond H, and the quinolone group were still detected in the microspheres system. In addition, there were interactions of Na alginate, $CaCl_2$, and maltodextrin as indicated by the presence of functional groups and the unification of OH stretching absorption from alginate with maltodextrin and the loss of guluronic fingerprints and an asymmetric carboxylic group uptake (1608 cm^{-1}) from Na alginate due to a crosslink with $CaCl_2$.

5.3. DTA Thermogram of Ciprofloxacin HCl-Alginate Microspheres

Results of DTA thermogram of Ciprofloxacin HCl-Alginate of F7 and F8 can be seen in Table 5.4.

Table 5.4.DTA thermogram of chemicals and microspheres

Sample	Melting Point (°C)
Na alginate	156,1 °C
$CaCl_2$	187,8 °C
Maltodextrin	173,9 °C
F7	176,5 °C
F8	183,2 °C

DTA Results of ciprofloxacin HCl-alginate microspheresof F7 and F8 in table 5.4 showed that each formula F7 and F8 had one endothermic peak. Interaction between sodium alginate polymer and $CaCl_2$ crosslinker was found because the melting point of the endothermic curve of all ciprofloxacin HCl-alginate microspheres was between the endothermic curves of sodium alginate and $CaCl_2$ (Nugrahani et al., 2013).

5.4. Particle size of Optimized Microspheres

Results ofparticle size of Ciprofloxacin HCl-Alginate of F7 and F8 can be seen in Table 5.5.

Table5.5 Particle size distribution of ciprofloxacin HCl-alginate microspheres of Formula 7

Class interval (µm)	Size average (d)	Amount of particle (n)	Procentage	n x d	n x d^2	n x d^3
1 - 1,36	1,18	108	36 %	127,44	150,38	177,45
>1,36 - 1,72	1,54	88	29,33 %	135,52	208,70	321,40
>1,72 - 2,08	1,90	45	15 %	85,50	162,45	308,66
>2,08 - 2,44	2,26	29	9,67 %	65,54	148,12	334,75
>2,44 - 2,80	2,62	10	3,33 %	26,20	68,64	179,85
>2,80 - 3,16	2,98	9	3 %	26,82	79,92	238,17
>3,16 - 3,52	3,34	2	0,67 %	6,68	22,31	74,52
>3,52 - 3,88	3,70	4	1,33 %	14,80	54,76	202,61
>3,88 - 4,24	4,06	2	0,67 %	8,12	32,97	133,85

>4,24 - 4,60	4,42	3	1 %	13,26	58,61	259,05
Number of particle		300	100 %	509,88	986,87	2230,30
dvs (µm)	2,26 µm					

Table 5.6 Particle size distribution of ciprofloxacin HCl-alginate microspheres of Formula 8

Class interval (µm)	Size average (d)	Amount of particle (n)	Procentage	n x d	n x d^2	n x d^3
1 - 1,35	1,18	89	29,67 %	104,58	122,88	144,38
>1,35 - 1,70	1,53	111	37 %	169,28	258,14	393,67
>1,70 - 2,05	1,88	59	19,67 %	110,63	207,42	388,92
>2,05 - 2,40	2,23	13	4,33 %	28,93	64,36	143,20
>2,40 - 2,75	2,58	12	4 %	30,90	79,57	204,89
>2,75 - 3,10	2,93	9	3 %	26,33	77	225,23
>3,10 - 3,45	3,28	2	0,67 %	6,55	21,45	70,25
>3,45 - 3,80	3,63	2	0,67 %	7,25	26,28	95,27
>3,80 - 4,15	3,98	1	0,33 %	3,98	15,80	62,81
>4,15 -4,50	4,33	2	0,67 %	8,65	37,41	161,80
Number of particle		300	100 %	497,05	910,31	1890,41
dvs (µm)	2,08 µm					

Table5.7 Polydispersity Index

Formula	Average of Particle Size	SD	PDI
F7	2,26	0,08	0,001
F8	2,08	0,17	0,010

Results showed particle size of ciprofloxacin HCl-alginate microspheres of F7 and F8. Observations also showed that the increased concentration of sodium alginate polymer, the size of microspheres particles increased. The PDI (Polydispersity Index) values of formulas showed the results of <0.1 indicated uniform and monodisperse particles (Wu et al., 2011).

Examination of microspheres showed that average particle size of ciprofloxacin HCl-alginate microspheres was F7 (2.19 ± 0.08); and F8 (2.08 ± 0.17). The results showed that the Na alginate contained was increased therefore the guluronic blocks crosslinked with more Ca^{2+} ions and formed egg box structure and particle size larger.

5.5 Moisture Content of microspheres

Moisture content of microspheres formula F7 and F8 can be seen in Table 5.8.

Table5.8.Moisture content of microspheres formula F7 and F8

Formula	Average ± SD (%)
F7	3,21 ± 2,12
F8	1,83 ± 0,68

Moisture content ofciprofloxacin HCl-alginate microspheres of F7 and F8showed that moisture content of microspheres of less than 10 %.

5.6 Swelling Index of Ciprofloxain HCl-alginate microspheres

Swelling indexof ciprofloxacin HCl-alginate microspheres of F7 and F8based on mass and particle sizecan be seen at table5.9 and 5.10.

Table 5.9. Swelling indexof microspheres based on mass

Formula	t = 12 hours	t = 24 hours
	Swelling index ± SD	Swelling index ± SD
F7	1,88 ± 0,37	2,33 ± 0,33
F8	1,79 ± 0,34	1,88 ± 0,66

Table 5.10. Swelling indexof microspheres based on particle size

Formula	t = 12 hours	t = 24 hours
	Swelling index ± SD	Swelling index ± SD
F7	2,06 ± 0,16	2,14 ± 0,19
F8	1,79 ± 0,35	1,95 ± 0,33

Swelling index indicated an increase of swelling index of ciprofloxacin HCl-alginate microspheres of F7 and F8 at12 hours and 24 hours.Swelling index data can be used to predict drug release from microspheres along with drug levels released per unit of time (Myrnes, 2016). The swelling index value to produce controlled release of the equilibrium state of alginate microspheres in phosphate buffer salts ranges from 1-2 (Chan et al., 2007; Shan et al., 2016).

5.7. Morphology of Microspheres using SEM

Morphology of ciprofloxacin HCl-alginate microspheres of formula F7 and F8 can be seen in Figure 5.1 and 5.2.

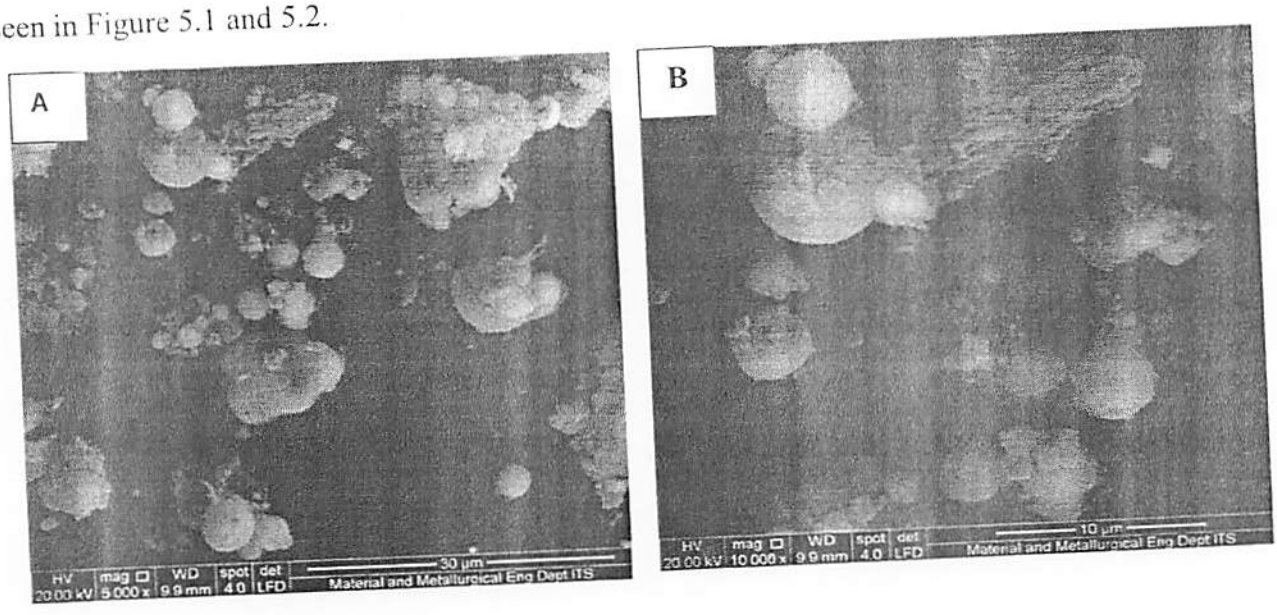


Figure 5.1. Morphology of ciprofloxacin HCl-alginate microspheres of formula F7

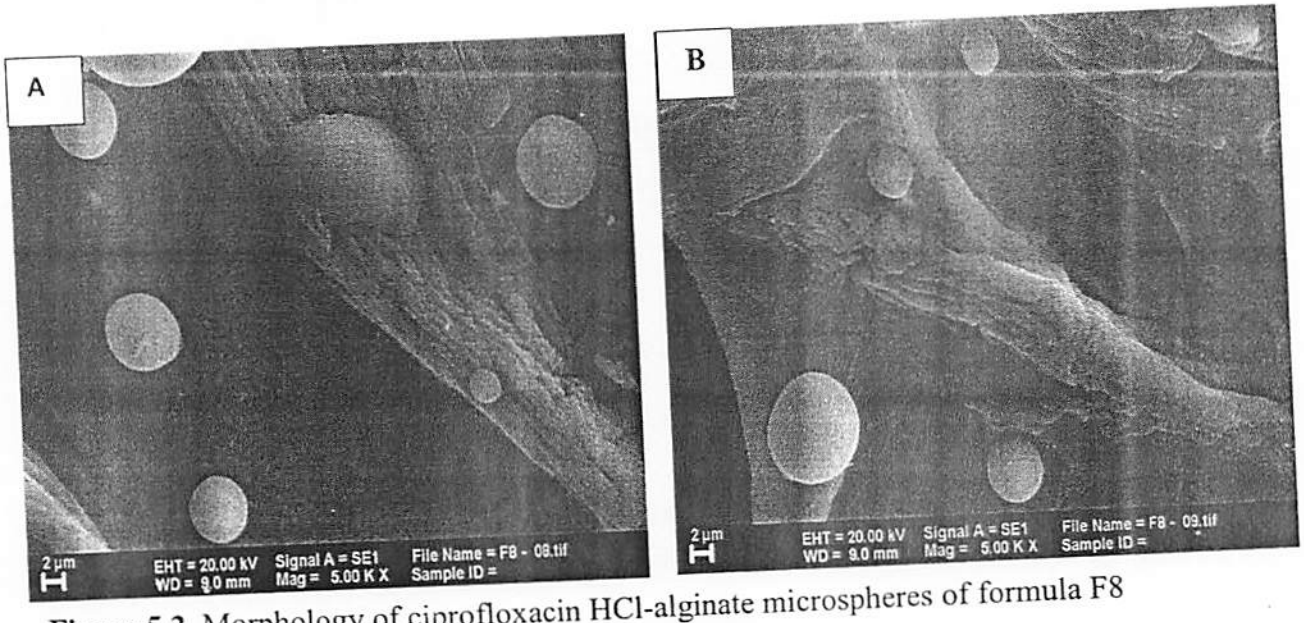


Figure 5.2. Morphology of ciprofloxacin HCl-alginate microspheres of formula F8

Observation results of particle surface morphology and shape using Scanning Electron Microscope (SEM) showed that microspheres produced were spherical with a smooth surface.

5.8. In vitro release of Ciprofloxacin –Alginate Microspheres

Results of in vitro release can be seen in Figure 5.3.

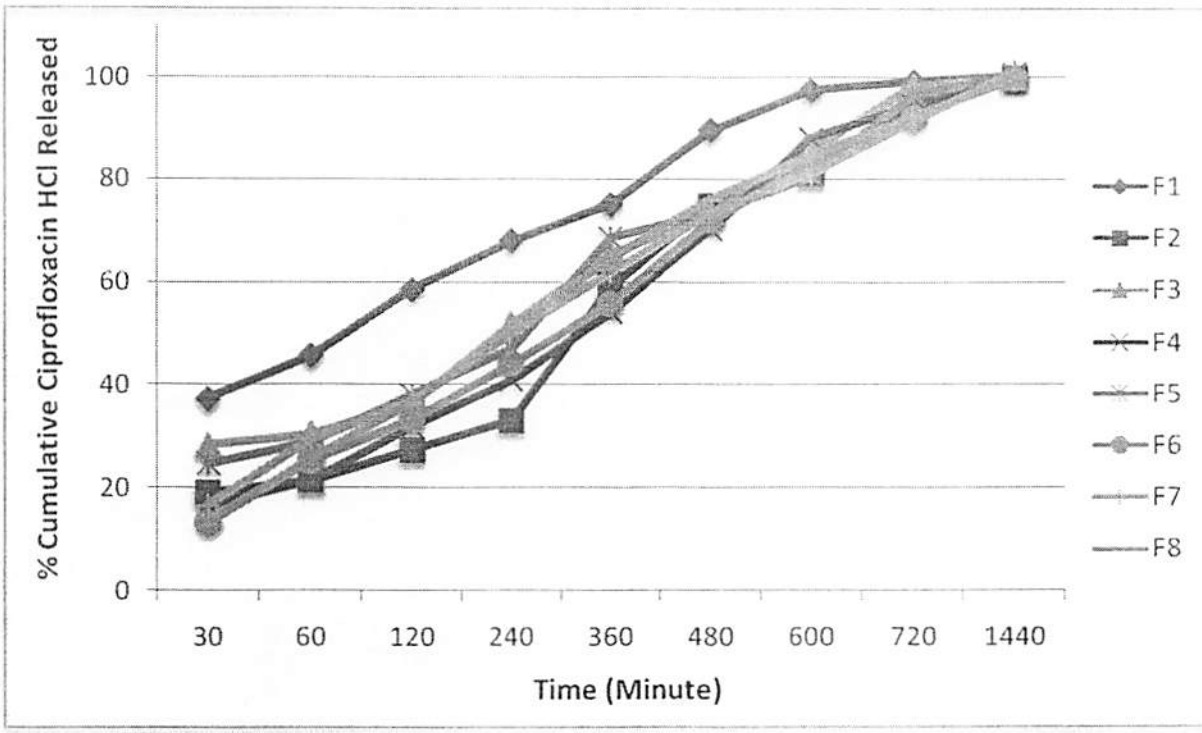


Figure 5.3. In vitro release study of microspheres

In vitro release study showed ciprofloxacin was released about 15-40% during first 30 minutes depends on the formula and continue for complete released in 12 hours. Moreover, the higher concentration of polymer and crosslinker, the slower the released. This in vitro release study showed that the microspheres produce sustained release for lung delivery that may results potency of ciprofloxacin as antibiotic released from alginate microspheres against microorganism for infection cystic fibrosis in lung disease.

5.9.Release Kinetics Evaluation

All the tested microspheres formulations of F1 to F8 were evaluated in terms of release kinetics and results as seen in Table 5.11.

Table 5.11. Release Kinetics of Microspheres

Formula	Orde Nol	Orde Satu	Higuchi	Korsmeyer-Peppas
F1	y= 0.000x + 0.095	y = 0.001x – 0.904	y = 0.029x – 0.088	y = 0.704x – 2.137
F2	y = 0.001x + 0.139	y = 0.001x – 0.797	y = 0.036x – 0.09	y = 0.666x – 1.931
F3	y = 0.001x + 0.146	y = 0.001x – 0.802	y = 0.043x – 0.134	y = 0.764x – 2.116
F4	y = 0.000x + 0.038	y = 0.001x – 1.233	y = 0.017x – 0.065	y = 0.708x – 2.421

F5	y = 0.000x + 0.094	y = 0.001x – 0.981	y = 0.020x – 0.033	y = 0.607x – 2.014
F6	y = 0.001x + 0.253	y = 0.001x – 0.556	y = 0.059x – 0.124	y = 0.646x – 1.656
F7	y = 0.001x + 0.285	y = 0.000x – 0.536	y = 0.033x + 0.066	y = 0.457x – 1.315
F8	y = 0.001x + 0.252	y = 0.001x – 0.580	y = 0.047x – 0.055	y = 0.600x – 1.605

Table 5.12. R^2 of Kinetics Release of Microspheres

Formula	Orde Nol	Orde Satu	Higuchi	Korsmeyer-Peppas
	R^2	R^2	R^2	R^2
F1	0.983	0.758	0.992	0.982
F2	0.977	0.758	0.992	0.985
F3	0.969	0.711	0.997	0.973
F4	0.993	0.829	0.976	0.993
F5	0.970	0.768	0.991	0.988
F6	0.966	0.762	0.996	0.990
F7	0.933	0.759	0.989	0.987
F8	0.953	0.746	0.994	0.986

All the tested microspheres formulations of F1 to F8 provided good fit to the Higuchi model. According to this model, the drug releasedfrom these batches may be controlled by diffusion through the micropores (16).

Ciprofloxacin HCl release from polymeric spheres can be explained by two mechanisms. The drug is released by diffusion from the encapsulating alginate microspheres. Secondly, the drug leaches out from the microspheres through the erosion and/or degradation of the matrix. The latter phenomenon could be attributed to the removal of the cross-linker, calcium, from the microspheres. The swelling of alginate molecules increases matrix porosity and thus increases both diffusion and erosion. These findings comply well with the higher drug to polymer ratio used in formulation (17). Phosphate buffer has a chelating action due to the phosphate ions which helps further in the disruption of the matrix. All formulations exhibited a sustained release of ciprofloxacin HCl over a period of 24 hours. A slower release pattern was observed for formulation containing higher amounts of the polymer, F7 and F8. Similar results were obtained for ropinirole hydrochloride loaded microspheres reported in a previous study (18). Similarly, insulin and diaminopyridine microparticles were successfully prepared by solvent evaporation method and drug to polymer ratio was shown to affect microspheres characteristics and drug release profile (19). From these results, it was aimed

for the microspheres system in this alginate matrix can be applied as a sustained release formula.

5.10. Minimum Inhibitory Concentration (MIC) of Ciprofloxacin HCl against *Staphylococcusaureus*

MIC results of ciprofloxacin HCl against*Staphylococcusaureus* were showed in table 5.13.

Table 5.13. MIC of ciprofloxacin HCl against *Staphylococcus aureus*

Concentration (ppm)	Inhibition ZoneDiameter (mm)			Average ± SD
	Replicate I	Replicate II	Replicate III	
2,00	19,90	18,95	18,40	19,08 ± 0,76
1,00	16,00	14,80	15,20	15,33 ± 0,61
0,50	11,55	11,50	11,25	11,43 ± 0,16
0,25	-	-	-	-
0,12	-	-	-	-
0,06	-	-	-	-

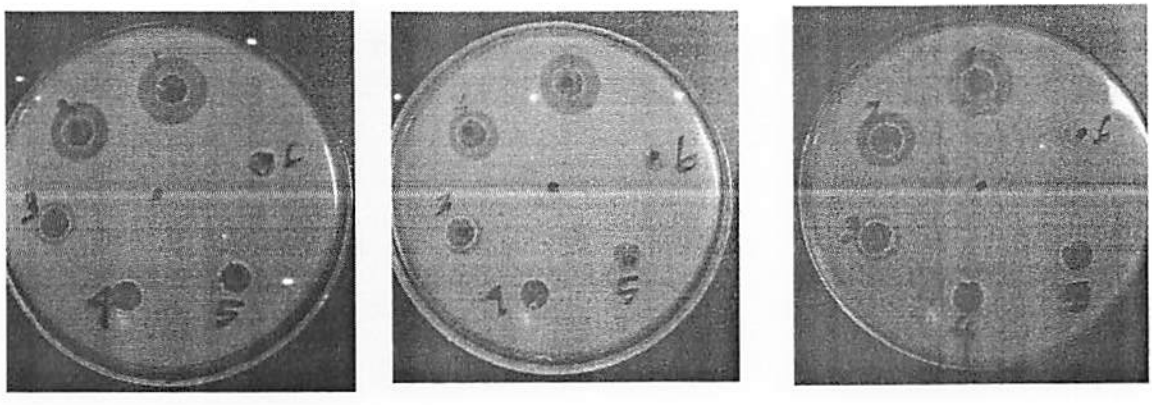


Figure 5.4. MIC ofciprofloxacin HCl against*Staphylococcus aureus*

5.11. Minimum Inhibitory Concentration (MIC) of Ciprofloxacin HCl of *Pseudomonas aeruginosa*

Table 5.14. MIC of ciprofloxacin HCl against*Pseudomonas aeruginosa*

Concentration (ppm)	Inhibition ZoneDiameter (mm)			Average ± SD
	Replicate I	Replicate II	Replicate III	
2,00	17,65	17,60	17,70	17,65 ± 0,05
1,00	14,45	14,60	14,10	14,38 ± 0,26
0,50	-	-	-	-
0,25	-	-	-	-
0,12	-	-	-	-
0,06	-	-	-	-

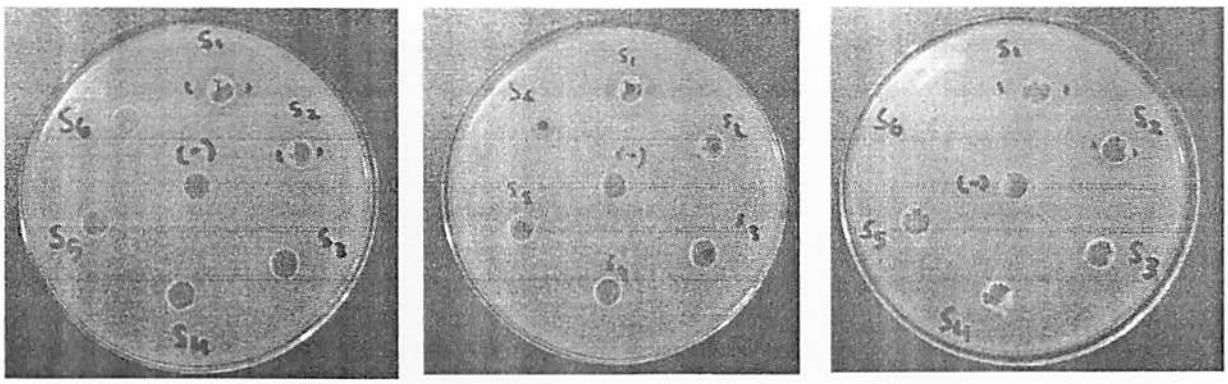


Figure 5.5. MIC of ciprofloxacin HCl against *Pseudomonas aeruginosa*

5.12. Antimicrobial Activity Assay Using Paper Disc Method

Results of Antimicrobial assay using paper disc methodcan be seen in Table 5.15.

Table 5.15. Antimicrobial assay of selected formula of ciprofloxacin HCl-alginate microspheres using paper disc method.

Sample Code	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
	Zone Diameter (mm)	Zone Diameter (mm)
Microspheres F7	6	6
Control (-)	6	6
Control (+)	6	6
Microspheres F8	6	15
Control (-)	6	6
Control (+)	6	6

From antimicrobial study, it can be seen that the activity between ciprofloxacin-alginate microspheres and standard ciprofloxacin control did not have a significant difference in activity. This means that ciprofloxacin HCl from encapsulated microspheres was not degraded during preparation formula and exposure of pH. This was because it was fully protected by microspheres delivery system; therefore ciprofloxacin activity was still high and protected from pH. Stability test results therefore suggested continuing with long-term storage testing. Furthermore, it is recommended to conduct an in vivo test to further refine this research.

5.13. Physical Stability Study of Ciprofloxacin HCl-Alginate Microspheres

Accordingstability test result on ciprofloxacin HCl stability evaluation at 25 °C and 40 °C temperature during storage period, 7 days, 14 days, 21 days and 30 days, it was obtained that the temperature and duration of the storage period give a significant effect on

ciprofloxacin HCl concentration. In the ciprofloxacin HCl stability test at 40 °C shown ciprofloxacin HCl concentration decreased with increasing times of storage after day 21(table 5.19). This was because ciprofloxacin HCl is one compound that is autooxidative and easily degraded at high temperature (Hagerman et al., 2003). For room temperature storage, it can be concluded microspheres drug delivery system in this study were proved to be able to maintain stability of ciprofloxacin HCl.

Result of physical stability test of this ciprofloxacin HCl-alginate microspheres had been in accordance with the results of stability evaluation conducted by Dashora (2007) at room temperature 25 °C ± 1 °C for 1 month. Dashora et al (2007) obtained results that microspheres remain stable at room temperature (Dashora et al., 2007). Stability test resultedat different temperature in terms of encapsulation efficiency and drug loading can be seen inTable 5.16.

Table 5.16. Physical Stability Test of Microspheres

Samples	Day	Drug Loading (%)		Entrapment Efficiency (%)	
		Room Temperature (25-27°C)	Accelerated Temperature (40°C)	Room Temperature (25-27°C)	Accelerated temperature (40°C)
F1	0	15.37000		14.81000	
	7	14.59344	14.72442	13.91947	13.91801
	14	14.70017	13.48389	13.52356	11.30294
	21	14.40457	6.60000	13.08071	6.00000
	30	14.62981	0.00000	13.46601	0.00000
F2	0	26.08000		22.45000	
	7	25.25568	20.70052	21.83520	20.50776
	14	24.69707	13.11668	20.45605	16.75026
	21	23.53460	7.76428	20.01908	1.46423
	30	23.05964	0.00000	19.89461	0.00000
F3	0	22.50000		23.39000	
	7	21.25489	20.76909	22.05874	9.22904
	14	20.25388	10.27840	23.04659	15.34080
	21	19.53059	5.06700	22.36711	5.75100
	30	19.06533	0.00000	22.78400	0.00000
F4	0	49.46000		56.43000	
	7	48.88988	40.67061	55.36709	40.06501
	14	48.60626	20.57710	54.09993	28.66235
	21	46.93158	9.80897	53.98301	14.84447
	30	45.94388	0.00000	53.16761	0.00000
F5	0	24.61000		25.51000	
	7	23.16688	20.90117	25.50316	23.51750
	14	22.73680	10.96315	24.05200	14.44734
	21	20.96107	4.40420	24.41604	6.06308
	30	20.67800	0.00000	22.54000	0.00000
F6	0	72.80000		94.40000	

	7	71.21091	62.44811	88.18795	46.77069
	14	71.09779	41.80942	86.48878	27.17755
	21	68.69672	11.23492	85.48479	18.54851
	30	60.18987	0.00000	82.85192	0.00000
F7	0	25.18000		39.64000	
	7	24.76783	20.55497	38.54816	28.34674
	14	21.16485	10.95983	37.51937	14.43589
	21	21.11692	5.56000	36.79846	4.356000
	30	21.05600	0.00000	36.4300	0.00000
F8	0	79.61000		95.29000	
	7	79.70017	61.84281	90.52356	87.64214
	14	78.62809	50.73051	89.42144	55.95771
	21	74.74579	10.86619	89.18689	12.99280
	30	70.43050	0.06034	88.07800	0.90516

CHAPTER VI
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Ciprofloxacin HCl-alginate microspheres were prepared successfully by using ionotropic gelation method by aerosolisation. Polymer concentration influenced the particle size as well as drug release pattern of microspheres. All formulas produced high yield and encapsulation efficiency and small size particles.The assessment of release kinetics showed that drug release from ciprofloxacin HCl-alginate microspheres followed the Matrix-Higuchi model (diffusion-controlled drug release mechanism). This formulation can bepotentially recommended for activity and stability test to further optimized as pulmonary drug delivery system.

For the selected best optimum ciprofloxacin HCl-alginate microspheres, high in vitro antimicrobial activity against Staphylococccus aureus and Pseudomonas aeruginosa were shown of formulas using high alginate and high $CaCl_2$ crosslinker concentration. However, after storage for 21 days at high temperature, the drug content of ciprofloxacin-alginate microspheres was reduced significantly showed instability at extreme condition. Therefore, storage information on the package label must be added by store at cool or room temperature.

6.2 Recommendations

Further evaluation of aerosol performance deposit using twin stage aerosols or cascade impactor equipment are highly recommended to simulate particle condition in the pulmonary.

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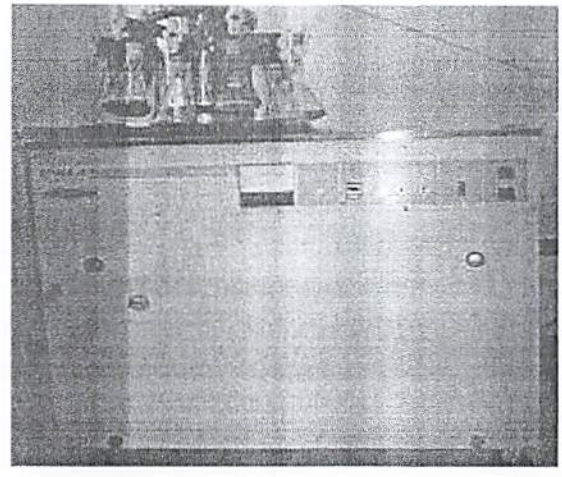
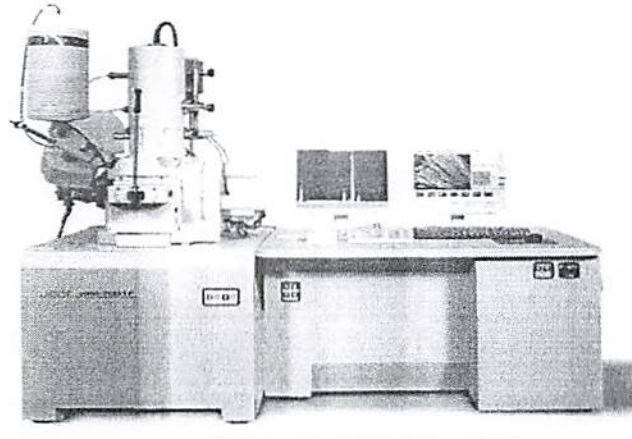
APPENDIX

APPENDIX 1. INSTRUMENT



Freeze Dryer



SEM

Moisture Content Analyzer

APPENDIX 2. RESEARCH TEAM ORGANISATIONAL AND JOB DESCRIPTION

1. Principal Researcher

Full Name	: Dewi Melani Hariyadi, SSi.,MPhil, Ph.D,Apt.
Sex	: P
NIP	: 197802262002122001
Discipline	: Pharmaceutics
Rank	: Penata Tk I/Gol IIId
Position	:Lektor/ Vice Dean of Research, Community Services and Collaboration
Faculty	: Pharmacy
Time Allocation	: 10 hour/week
Job description	: Coordinator and research designer, formulation, evaluation in vitro, stability study, data analysis, drafting manuscript.

2. Member of Researcher

Full Name	: Dra. Esti Hendradi,MSi, PhD, Apt.
Sex	: P
NIP	: 195711141987032001
Discipline	: Pharmaceutics
Rank	: Pembina/ Gol.IVA
Position	:Lektor Kepala
Faculty	: Pharmacy
Time Allocation	: 8 hour/week
Job description	: formulation alginate microspheres, optimization technique, Effectivity antimicrobial and antibiotic potency.

3. Member of Researcher

Full Name	: Dr. Nazrul Islam
Sex	: L
NIP	: -
Discipline	: Pharmaceutics
Rank	: -

Position	: Senior Lecturer
Faculty	: Pharmacy
Time Allocation	: 8 hour/week
Job description	: Dispersibility test, expertise in lung delivery and drafting manuscript

APPENDIX 3. ARTICLE

1. UNDER REVIEW JURNAL YEAR 1: FABAD Journal of Pharmaceutical Sciences (INTERNATIONAL JOURNAL SCOPUS): Physical Characterization of Ciprofloxacin HCl Encapsulated in Ca-Alginate Microspheres at Different Concentration of Alginate and CaCl2 as Pulmonary Delivery System (Dewi M Hariyadi et al, 2018)

Physical Characterization of Ciprofloxacin HCl Encapsulated in Ca-Alginate Microspheres at Different Concentration of Alginate and $CaCl_2$ as Pulmonary Delivery System

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ABSTRACT

Inhalation treatment using antibiotic is an alternative for lung delivery especially for cystic fibrosis, however, the therapeutic efficacy of inhaled drugs is limited by their rapid clearance in the lungs. Sustained release system in the lungs can improve therapeutic outcomes of inhaled drugs because they can retain the drug load within the lungs and progressively release the drug locally at therapeutic levels. This study presents the formulation strategies developed to control drug release in the lungs using alginate polymer-based microspheres system. The microspheres composition can be adjusted to modulate drug release and can encapsulate compounds with high drug loading. Pulmonary route is commonly used and has been well accepted as a portal for non-invasive drug delivery for many lung diseases and it is explored for decades as an alternative for systemic as well as local drug delivery. The present study explores the in vitro benefits of ciprofloxacin antibiotic encapsulated alginate microspheres. The in vitro studies include size, shape, morphology, yield, drug loading and encapsulation efficiency.

Current results showed small, smooth and spherical ciprofloxacin-alginate microspheres were produced using aerosolization technique. Small particles of less than 5μm were formed which suitable for inhalation aerosol particles for lung or pulmonary delivery. High entrapment efficiency up to 95%, loadings of 80% and yield of 85% were also showed from the microspheres. The most promising physical characteristics of microspheres for pulmonary delivery will need further evaluation of the potency against microorganism in lung disease.

Keywords: alginate, microspheres, ciprofloxacin HCl, cystic fibrosis, pulmonary delivery.

1. INTRODUCTION

The increase of lung treatment especially in cystic fibrosis has gained attention in the last decade [1]. Drug delivery for lung treatment in the form of microspheres offer an alternative to deliver high drug concentration directly to the site of action to improve the therapeutic effect and minimize the side effect [1,15].Active agent to be used as model for lung delivery in cystic fibrosis is antibiotic group with microspheres delivery system as a promising approach for antibiotic inhalation. Infection which usually occurred in the lung delivery is cystic fibrosis.

Cystic fibrosis (CF) is a genetic disorder that affects mostly the lungs but also the pancreas, liver, kidneys, and intestine [15]. CF is most common among people of Northern European ancestry and affects about one out of every 3,000 newborns. About one in 25 people are carriers. It is least common in African and Asians [13].

Long-term issues include difficulty breathing and coughing up mucus as a result of frequent lung infections [14]. Other signs and symptoms include sinus infections, poor growth, fatty stool, clubbing of the fingers and toes, and infertility in males, among others. Different people may have different degrees of symptoms. Lung infections are treated with antibiotics which may be given intravenously, inhaled, or by mouth. Sometimes the antibiotic azithromycin is used long term. Lung transplantation may be an option if lung function continues to worsen.

About 80% of adults with cystic fibrosis have chronic infections of Pseudomonas aeruginosa and 50% caused death within 5 years [2,3,4], where Staphylococcus aureus and Haemophylus influenza are primarily pathogenic in children [4]. In isolation of saliva cultures, cystic fibrosis patients can be infected with Haemophilus influenza, Staphylococcus aureus, Pseudomonas aeruginosa, Burkholderia cepacia, Escherichia coli and Klebsiella pneumonia [5]. Treatment of lung disease using antibiotics is to reduce infection and control inflammation [6] To enhance drug efficacy and reduce the dose and side effects, microspheres are alternative of lung delivery system, particularly in cystic fibrosis. Chemical properties of forming polymer may provide potential efficacy for antibiotic drug. The microspheres composition can be adjusted to modulate drug release and can encapsulate compounds with high drug loading.

Alginate-based microspheres provide sustained release properties with several advantages such as minimum usage of toxic organic solvents and reduced reticuloendothelial system uptake due to stealth nature of alginate. The present study explores the therapeutic benefits of antibiotic encapsulated alginate microspheres when administered by pulmonary route animal model as well as in vitro physical evaluation. The encapsulation method is by aerosolization technique considered as simple, easy, and produces small and uniform particle size.

The microspheres are spherical monolithic or agent therapeutic distributed in the matrix either as a dispersion of molecular or particle, or can be defined as a structure consisting of a continuous phase of one or more soluble polymer dispersed at the molecular level or macroscopic [17]. Microspheres have a particle size of 1-1000 nm [18]. In the field of pharmaceuticals, drug delivery systems with technology microspheres used for the preparation of slow release and controlled, reduce and even eliminate the irritation of the gastrointestinal tract, protect the drug from the ravages and support the spread of drugs distributed in the gastrointestinal tract resulting in absorption of the drug is more reproducible [7]. The smaller the drug particle size, the greater the absorption in the gastrointestinal tract [16].

Some of the methods used for preparation of microspheres are emulsion solvent evaporation, continued cross-emulsion, conservation thermal changes, spray drying, solvent diffusion emulsion, and gelation ionotropic. Ionotropic gelation technique is a method of preparation of microspheres by adding the drug solution into the polymer solution and the solution of the crosslinking agent, and item the process gelification for 24 hours [7]. The advantage of using ionotropic gelation method in the preparation of the microspheres is able to maintain drug integrity so that the drug can be encapsulated without the use of organic solvents or elevated temperatures; this causes the drug to remain stable. In addition, the gelation ionotropic method was quite simple, fast, and cost-effective [8].

Advantages of using Na-Alginate are biocompatible, biodegradable, non-toxic and has been recognized for its safety by the

2. INTERNATIONAL CONFERENCE ICoAPS: ANTIMICROBIAL ACTIVITY OF CIPROFLOXACIN HCl-ALGINATE MICROSPHERES (Against Staphylococcus aureus and Pseudomonas aeruginosa)AT DIFFERENT CONCENTRATION OF ALGINATE POLYMER: Dewi M Hariyadi et al, Husnia, 2018 (POSTER PRESENTER)

Ciprofloxacin HCl is a broad-spectrum second generation floroquinolone antibiotic that is not susceptible to bacterial resistance that can be used to treat infections in the lower respiratory tract. One infection in the lower respiratory tract is anthrax and cystic fibrosis. Increased viscosity of pulmonary mucus in cystic fibrosis patients causes bacteria to attach easily and accumulate and absorb into the mucin. The two bacteria that occupy the top ranks are Staphylococcus aureus and Pseudomonas aeruginosa. In this study, microspheres can be used to entrap active agent, improve biocompatibility, thereby reducing the risk of immunogenicity and toxicity and increase bioavailability and ability to produce sustained releases.

This study aims to determine the optimum formula of microspheres, evaluate in vitro release, release kinetics, antimicrobial activity and physical stability of ciprofloxacin HCl - alginate microspheres. Physical characterization includes organoleptic, shape, and surface morphology of microspheres. Based on the physical identification of ciprofloxacin HCl-alginate microspheres, it was found results in accordance with the literature.

Antibacterial activity test was carried out in vitro using Staphylococcus aureus and Pseudomonas aeruginosa bacteria. Results showed no significant difference between ciprofloxacin HCl activitiesof microspheres compared to positive control. Furthermore, the encapsulation process did not affect changes in encapsulation efficiency and drug loadings of ciprofloxacin HCl. Long-term stability testing in storage and in vivo testing was recommended in further research.

3. SUBMITED MANUSCRIPT JURNAL YEAR 2 (JOURNAL OF PHARMACY AND BIOALLIED SCIENCE): (INTERNATIONAL JOURNAL SCOPUS Q3): Release Profile, Kinetics and Activity of Ciprofloxacin HCl-Alginate Microspheres (Dewi M Hariyadi et al, 2018)