DESIGN AND ANALYSIS OF LYOPHILIZED FORMULATION FOR EXTENDING SHELF LIFE OF TUBERCULIN PPD

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DESIGN AND ANALYSIS OF LYOPHILIZED FORMULATION FOR EXTENDING SHELF LIFE OF TUBERCULIN PPD.

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ABSTRACT

Tuberculin test is currently used for diagnosis and epidemiological surveys of tuberculosis. Mantoux method involves the intradermal injection of purified protein derivative (PPD) at the International unit dose. This study aimed to develop a dry PPD preparation to extend the shelf life of tuberculin PPD. Therefore, 5.0% Sucrose (S), 6.5% Mannitol (M), 2.5% Trehalose (T) and 0.3% Haemaccel (H) were added to each formulation. *In vivo* and *in vitro* analyses were carried out after lyophilization in order to determine the efficacy of the tuberculin activity.

In the *in vivo* test, the activities of tuberculin shown by Delayed type hypersensitivity (DTH) responses to lyophilized preparations were compared to DTH responses to liquid preparation (CL) after injection into Bacillus Calmette Guerin (BCG) vaccinated guinea pigs. The results from preparations with added H, M, T, and S were 100%, 90%, 89%, and 60% of those from CL respectively. There was no loss of tuberculin activity in H formula. A statistically significant difference was found between S and CL ($P < 0.05$). The cellular test for Interferon-gamma (IFN-γ) secretions was performed by using whole blood of human subjects screened for having DTH responses to tuberculin PPD Mantoux tests. They were assayed for Cell mediated immunity (CMI) responses caused by the stimulation of tuberculin antigens in the formulations. The detection of IFN-γ secretions was done by using Enzyme-Linked Immunosorbent Assay and the efficacy was expressed in terms of percentage of IFN-γ responses to tuberculin antigens. The results of CL, H, M, T and S were 3.28%, 10.40%, 0.84%, 1.52% and 1.29% respectively. The lyophilized H, M and T formulations and the liquid CL were studied for their shelf-life stabilities. Accelerated degradation was induced by storing the samples at 4°C and higher temperatures of 37°C and 56°C for 3, 6, 9 and 12 months. All tuberculin PPD solutions were injected into BCG vaccinated guinea pigs at the end of each storage period and the activities of tuberculin were evaluated. From the graphical profile of the remaining activities and the storage times, Haemaccel was superior to the other excipients especially at the normal storage temperature of 4°C for long term of 12 months.

The results demonstrate the effectiveness of Haemaccel. It provided good stability and may lead to long shelf life. This research supports the potential of development of lyophilized tuberculin PPD with the addition of 0.3% Haemaccel.

KEY WORDS: TUBERCULIN PPD/ MANTOUX TEST/ DTH RESPONSE/ CMI RESPONSE/ IFN-γ SECRETION

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การสร้างและวิเคราะห์สูตรตำรับของทูเบอร์คูลินพีพีดีชนิดแห้งเพื่อเพิ่มอายุในการใช้งาน
(DESIGN AND ANALYSIS OF LYOPHILIZED FORMULATION FOR EXTENDING SHELF LIFE OF TUBERCULIN PPD)

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บทคัดย่อ

การทดสอบการติดเชื้อวัณโรคด้วยทูเบอร์คูลินในปัจจุบันยังนิยมใช้วิธี Mantoux ที่ใช้ทูเบอร์คูลินชนิด purified protein derivative (PPD) ฉีดเข้าชั้นผิวหนังตามขนาดฉีดมาตรฐาน ซึ่งน้ำยามีความเจือจางสูงของปริมาณโปรตีน ทำให้ไม่มีความคงทน อายุของน้ำยาสั้น งานวิจัยนี้มีวัตถุประสงค์ในการพัฒนาสูตรตำรับทูเบอร์คูลิน PPD เป็นชนิดแห้งเพื่อยืดอายุการใช้งานและการเก็บรักษา โดยเติม 5.0% Sucrose (S) 6.5% Mannitol (M) 2.5% Trehalose (T) และ 0.3% Haemaccel (H) ลงในแต่ละสูตรตำรับ หลังจากผ่านกระบวนการทำแห้ง (lyophilization) ได้นำตัวอย่างของแต่ละตำรับไปวิเคราะห์ประสิทธิภาพของทูเบอร์คูลินคงเหลือ วิธีทดสอบในสัตว์ทดลองได้แก่การหาปริมาณโปรตีนในแต่ละสูตรที่สามารถกระตุ้น DTH response ซึ่งแสดงผลด้วยขนาดปฏิกิริยาที่ผิวหนังจากการฉีดตัวอย่างทูเบอร์คูลิน PPD เข้าผิวหนังหน้าท้องหนูตะเภาที่ฉีดกระตุ้นด้วยวัคซีนบีซีจีไว้แล้ว ขนาดเฉลี่ยของปฏิกิริยาตอบสนองต่อทูเบอร์คูลินชนิดแห้งแต่ละสูตรเปรียบเทียบกับขนาดของปฏิกิริยาตอบสนองต่อทูเบอร์คูลินชนิดน้ำ (CL) ได้ผลเรียงตามลำดับคือ H M T และ S เท่ากับ 100% 90% 89% และ 60% สูตร H ไม่มีการสูญเสียของปริมาณโปรตีนหลังการทำแห้ง และผลระหว่าง S และ CL มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) เมื่อทำการทดสอบ IFN-γ secretion จากเลือดของผู้ที่ได้รับการทดสอบแล้วว่ามี CMI response ต่อทูเบอร์คูลินด้วยวิธี Mantoux เพื่อหาปริมาณสาร IFN-γ ที่หลั่งจากการกระตุ้นเม็ดเลือดขาวด้วยทูเบอร์คูลินแอนติเจนตามปริมาณที่มีอยู่ในแต่ละสูตร พบว่าระดับ IFN-γ จากสูตร CL H M T และ S เท่ากับ 3.28% 10.40% 0.84% 1.52% และ 1.29% ตามลำดับ ซึ่งประเมินได้ว่าสูตร H สามารถกระตุ้นให้มีการหลั่งสาร IFN-γ ได้มากที่สุด การทดสอบความคงทนของทูเบอร์คูลินชนิดแห้งเฉพาะสูตร H M T ซึ่งมีปฏิกิริยาตอบสนองต่อทูเบอร์คูลินในหนูตะเภาไม่แตกต่างจากสูตร CL โดยวิธีวิเคราะห์ความคงสภาพของโปรตีนแบบเร่งการสลายตัวด้วยการเพิ่มอุณหภูมิและเวลา (accelerated degradation) ได้เก็บตัวอย่างของ H M T และ CL ที่ 37°C 56°C และ 4°C เป็นเวลา 3 เดือน 6 เดือน 9 เดือนและ 12 เดือน เมื่อครบแต่ละช่วงเวลาที่กำหนดได้ทดสอบหาปริมาณโปรตีนคงเหลือในแต่ละสูตรที่สามารถกระตุ้นปฏิกิริยาตอบสนองแบบ DTH ในหนูตะเภา ผลที่ได้แสดงว่าสูตร H สามารถรักษาปริมาณคงเหลือของโปรตีน PPD ได้มากกว่าสูตรอื่น โดยเฉพาะการเก็บที่ 4°C ในเวลา 12 เดือน ผลของการวิเคราะห์ตัวอย่างที่เก็บรักษาแบบปกติร่วมกับการทดสอบความคงทนแบบเร่งการสลายตัวจากงานวิจัยนี้ทำให้ประเมินได้ว่า สูตรตำรับที่มีการเติม 0.3% Haemaccel สามารถนำไปพัฒนาเป็นสูตรตำรับของทูเบอร์คูลินพีพีดีชนิดแห้งที่มีอายุการเก็บรักษาในอุณหภูมิ 4°C ได้ยาวนานกว่าสูตรอื่นและทูเบอร์คูลินชนิดน้ำ

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LIST OF ABBREVIATIONS

ADT	**Accelerated degradation test**
AFB	**Acid-fast bacillus**
Ag	**Antigen**
APC	**Antigen presenting cell**
BCG	**Bacillus Calmette Guerin**
CD	**Control of dried tuberculin**
CL	**Control of liquid tuberculin**
CMI	**Cell mediated immunity**
DNA	**Deoxyribonucleic acid**
DTH	**Delayed type hypersensitivity**
EH	**Excipient Haemaccel**
ELISA	**Enzyme-Linked Immunosorbent Assay**
EM	**Excipient Mannitol**
ES	**Excipient Sucrose**
ET	**Excipient Trehalose**
g	**gram**
H	**Haemaccel**
HIV	**Human Immunodeficiency Virus**
HMI	**Humoral immunity**
hr	**hour**
IFN-γ	**Interferon gamma**
IU	**International unit**
LAM	**Lipoarabinomannan**
M	**Mannitol**
mbar	**millibar**
mg	**milligram**
min	**minute**

LIST OF ABBREVIATIONS (cont)

ml	**millilitre**
mm	**millimetre**
Nc	**Negative control**
NIH	**National Institute of Health**
nm	**nanometre**
No.	**Number**
OD	**Optical density**
PBS	**Phosphate buffer saline solution**
Pc	**Positive control**
PCR	**Polymerase chain reaction**
PPD	**Purified protein derivative**
PPD-S	**Standard Purified protein derivative**
ppm	**part per million**
QSMI	**Queen Saovabha Memorial Institute**
Ref	**Reference**
S	**Sucrose**
T	**Trehalose**
TRC	**Thai Red Cross**
TB	**Tuberculosis**
TU	**Tuberculin unit**
U.S.	**United State**
WFI	**Water for injection**
^{o} C	**Degree Celsius**
µg	**microgram**
µl	**microlitre**
µm	**micrometre**

CHAPTER I
INTRODUCTION

Tuberculosis is a serious public health problem relating to the spread of *Mycobacterium tuberculosis* which is a highly infectious pathogen that can cause death world-wide particularly in low socioeconomic countries. Tuberculin test is currently used for screening in diagnosis and epidemiological surveys of tuberculosis.

Tuberculin is a liquid preparation from killed *Mycobacterium tuberculosis*. Purified protein derivative (PPD) of tuberculin is required as the reagent for Mantoux test procedure which is the general test for quantification of the delayed type hypersensitivity (DTH) reaction in the infected subject. Potency or activity of tuberculin can be assayed by biological test which is an estimation of the protein content in the solution and measuring the response of intradermal injection into BCG vaccinated guinea pigs. Responsiveness due to DTH results in a localized induration at injection site. The diameter of reaction observed in guinea pig is generally measured at 24 hours after injection (1). In the standard Mantoux test in human, 0.1ml of diluted tuberculin solution containing 5 IU of PPD is injected intradermally on the volar surface of the forearm and the size of induration is read 48 to 72 hours later (2).

In the past, tuberculin testing has been restricted to perform in a small amount by using freshly prepared of diluted tuberculin PPD. At present, the product of diluted tuberculin that are ready for use in Mantoux test has been largely requested from users in the hospitals and the tuberculosis control programs. The increasing of usage come from a number of screening tests for tuberculosis in the era of HIV infections. The ready for use product of tuberculin PPD has become very necessary for public health services since it has been reported in certain cercumstance that dilutions of tuberculin PPD lost about 20% of the potency in 24 hours. However, their potency could be maintained up to only 6 months during storage at 2-4 ^{o}C (3). The duration of 6 months after production includes the time required for quality control tests and shipment of the products, consequently the actual shelf life of diluted PPD in hands of the users is about 4 or 5 months that is inadequate when compared to the shelf life of other

pharmaceutical products. The tuberculin PPD when highly diluted for use results in a short shelf life and remains a serious problem of the producers and the users. Accordingly, the question whether ready for use dilution of Tuberculin PPD can withstand prolonged storage has therefore become very important.

In Thailand, the diluted Tuberculin PPD products for use in DTH skin tests of Mantoux method are produced at QSMI laboratory of the Thai Red Cross, namely TRC-Tuberculin PPD and distributed to the users throughout the country. The concentrate solution of Tuberculin PPD is diluted to 100 units per ml or 10 units per dose. The strength of 10 unit dose of diluted tuberculin PPD produced by QSMI is equivalent to 5 IU dose of the International Reference Standard PPD or PPD-S (4).

The practice of production has focused on maintaining the activity of tuberculin in diluted form. The centralized preparation can reduce the risk of contamination and gross error, and permits standardization of the dilutions. At QSMI laboratory, the tuberculin PPD dillution is routinely prepared every month because its activity becomes unstable after dilution. The shelf life of liquid tuberculin PPD is now definitely limited by the manufacturer at 6 months. Each new batch of dilution have to be standardized in BCG sensitized guinea pigs against the preceding batch (5).

In this research, the study for extending shelf life of diluted tuberculin PPD is focused on the development of tuberculin PPD products by addition of protective excipients in order to protect proteins through lyophilization process. Four different kinds of excipients; Sucrose (S), Mannitol (M), Trehalose (T) and Haemaccel (H) were designed for suitable contents and added to each formulation. After lyophilization, analysis has been performed in order to study the effectiveness of those excipients used for protection of proteins in diluted solution that corresponded to the amount of remaining tuberculin activities in the formulations. In biological assay, the results of tuberculin activities shown by DTH responses after injections of tuberculin PPD solutions into the skin of BCG vaccinated guinea pigs were compared to the initial activity of liquid preparation (CL). The cellular response tested is by using IFN-γ immunoassay which has been developed for quantification of IFN-γ secretions in plasma of tuberculin skin test-positive individuals (6). Blood from human subjects screened for DTH responses to tuberculin PPD Mantoux tests are used to determine the level of IFN-γ secretions following a stimulation of tuberculin antigen in each

formula. Finally, the study for shelf-life stabilities of the developed products is done by accelerated degradation at various conditions of high temperatures and storage time durations.

Development of the lyophilized formulation for extending shelf life of tuberculin PPD will be valuable for the well-known tuberculosis diagnosis, tuberculin skin test, which remains not to be replaced by any other test at present. The successful design of formulation and lyophilization program may lead to the effective methods for maintaining the activity of tuberculin PPD and being able to achieve longer shelf life of the product.

Objectives

The specific objectives of this study are the followings:

1. To extend the shelf life of diluted tuberculin PPD by designing the lyophilized formulation with addition of appropriate excipients
2. To compare the tuberculin activity of lyophilized formulations with liquid formulation by using *in vivo* and *in vitro* analyses
3. To study the stability of the ready for use product of lyophilized tuberculin PPD for Mantoux skin test.

CHAPTER II
LITERATURE REVIEW

Tuberculosis is an important infectious disease that remains a major problem of public health and ranks among the top ten causes of death in the world. About one-third of the world's population is infected with *Mycobacterium tuberculosis*. Every year, approximately 8 million people develop active disease, 2-3 million people die of tuberculosis; mostly in developing countries and over 400,000 new cases found in industrialized country (7, 8). The tuberculosis patients usually present with pulmonary disease; well known symptoms are chronic cough, low-grade fever, night sweats and weight loss. They may have extra pulmonary manifestations including lymphadenitis; kidney, bone or joint involvement, meningitis or disseminated disease. Lymphadenitis and meningitis are commonly found in tuberculosis infected infants, and all extra pulmonary manifestations are increased in frequency among immunocompromised individuals such as patients on chronic renal dialysis, elderly, malnourished and HIV-infected individuals. Persistent infection may reactivate after decades owing to deterioration of immune status (9). In order to control the spread of tuberculosis, it is necessary to identify and perform treatment for infected persons before they become infectious to other persons through the progression of the disease. One essential factor for controlling the spread of tuberculosis is the ability to diagnose infection in its early stages. Acceptable diagnosis and treatment could lead to decreases in the disease.

1. *Mycobacterium tuberculosis* and infection

Robert Koch, a German physician discovered the tubercle bacillus, *Mycobacterium tuberculosis* which was the cause of tuberculosis. In 1890, he announced the discovery of a cure for this disease that consisted of giving patients subcutaneous doses of tuberculin, a brownish, transparent liquid obtained from culture filtrates of *M. tuberculosis* (2).

1.1 *M. tuberculosis* structure

Mycobacteria are slender curved non-motile rods (1-4 μm length) and have no spore formation. This microorganism is known as acid-fast bacillus (AFB) as shown in Figure 1. Cell wall is composed of mycolic acids, complex waxes, sulfatides, unique glycolipids and arabinogalactan (Figure 2). This unusual cell wall gives a particular quality of resistance to dehydration and decolorlization with acid alcohol after staining with carbol-fuchsin. Cell membrane associated with lipoarabinomannan (LAM) which affects expression of cytokines by macrophages (9). The lipid containing cell wall has the ability to protect the cell from adverse environment as found within the host tissue. The other effect of lipid cell wall is that it can induce certain activities, which assist the host to progress the disease and destroy the tissue (10).

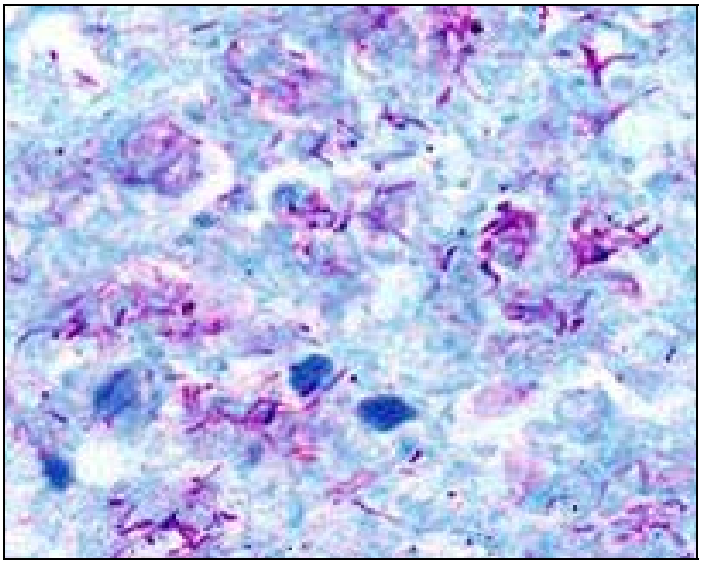

Figure 1 Rod-shaped bacteria, *Mycobacterium tuberculosis* are acid-fast bacilli (shown in red) (11)

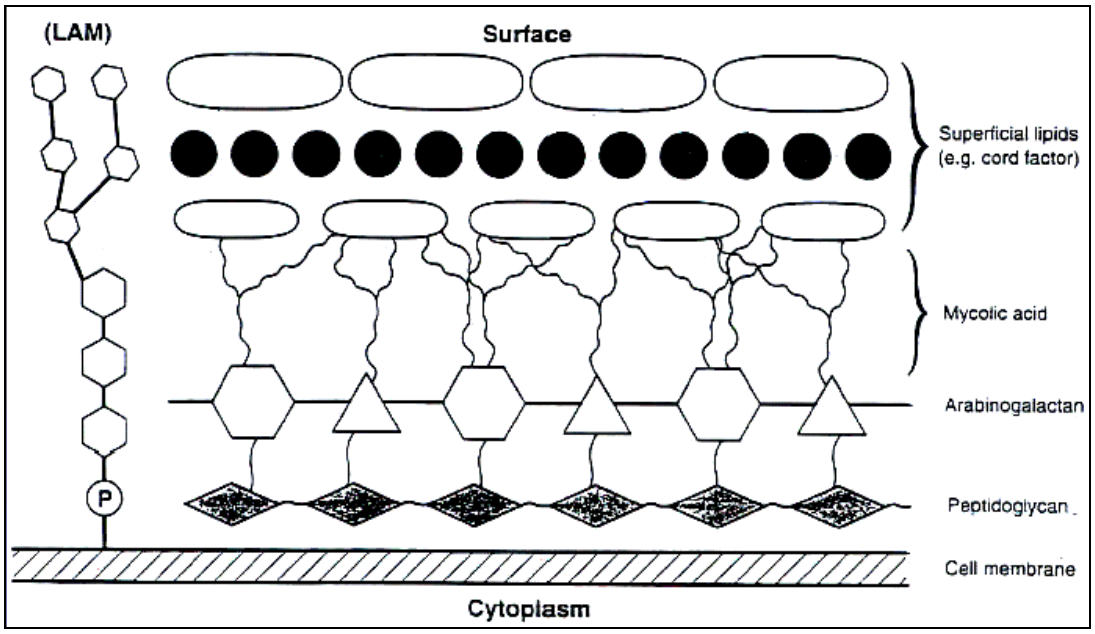

Figure 2 Cell wall structure of *Mycobacterium tuberculosis* (9)

1.2 Infection and pathogenesis in human

M. tuberculosis is transmitted by the respiratory route. It is capable of causing disease in most organs, pulmonary tuberculosis is the most common. The important source of tuberculosis is the already infected person who spreads the highly virulent bacilli via respiratory droplets. Children are often infected with mycobacterial bacilli in the area of high incidence and high population density. Primary infections of *M. tuberculosis* can occur in adults and children at any age. Although after treatment of the disease, the tubercle bacilli can be reactivate and spread again. Usually, primary infection has no symptom and often resolves spontaneously. The infections progress by localized infection and spread in the lungs to cause pleurisy and bronchopneumonia (Figure 3). Early in infection, mycobacteria may spread either indirectly through the lymphatics to the lymphnodes and hence via the thoracic duct into the blood stream or directly into the circulation by erosion of the developing tubercle into a pulmonary vessel. In systemic infection, the pathogens spread into blood stream, they can affect many organs, including the meninges, the bones or the internal organs and eventually reinfect the lung (8, 9). In case of active tuberculosis, a heavy bacterial load and concomitant high levels of circulating antibody results from the inability of the immune system to contain bacterial growth (6).

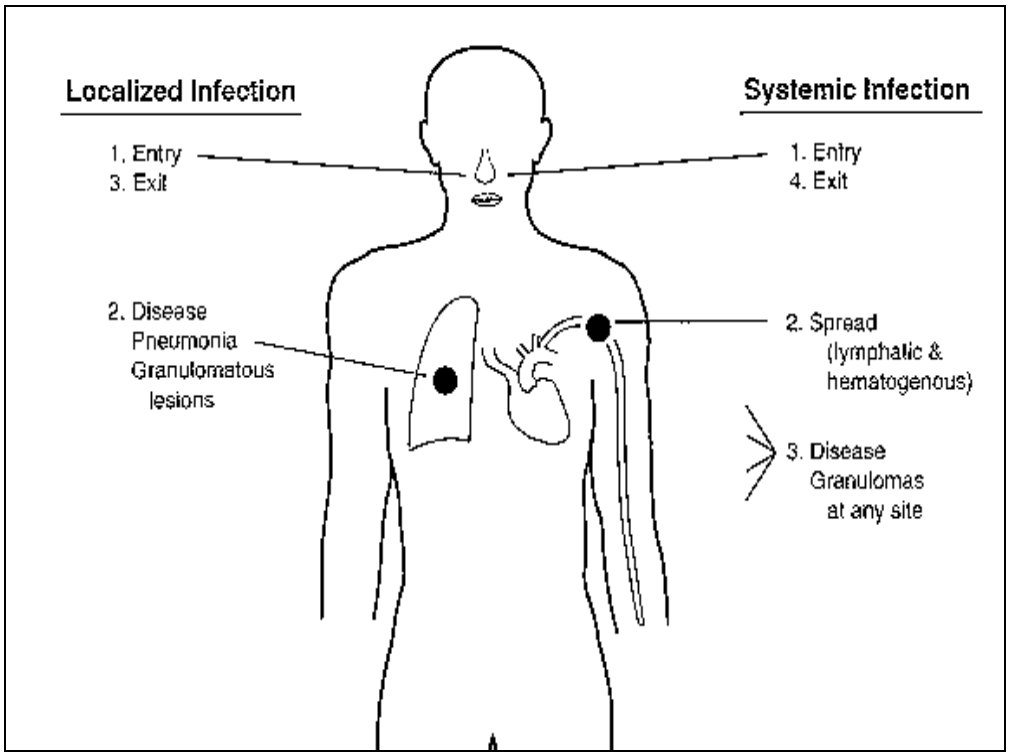

Figure 3 Infection and pathogenesis of tuberculosis in human (9)

1.3 Diagnosis of tuberculosis

The infection in asymptomatic individual can be diagnosed by intradermal injection of tuberculin PPD into the volar surface of the forearm, Mantoux method (Figure 4). The injection of tuberculin PPD into a previously infected person results the positive reaction of delayed hypersensitivity with an induration at the site of injection. A diagnosis of active disease is based on symptoms, an abnormal chest radiograph, acid fast staining for mycobacteria in sputum or bronchial specimens (9). PCR assays based upon DNA amplification of mycobacterial genes have currently been used for rapid identification in clinical specimens (12).

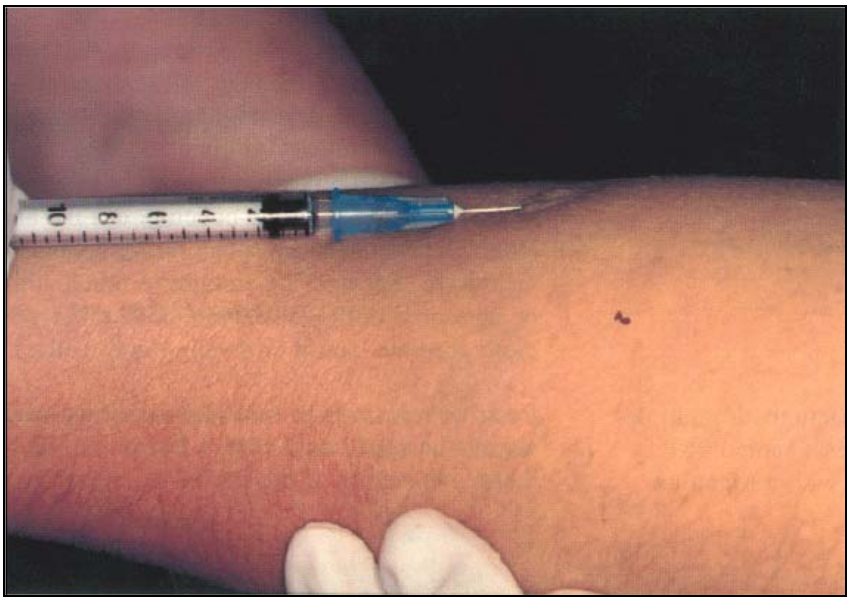

Figure 4 Tuberculin PPD-Mantoux skin test injection (13)

2. Host defenses and immune responses to infection

The tubercle bacillus contains a number of proteins or polypeptides which play important roles in the response to infection. Primary infection can induce both immune and nonimmune inflammatory reaction. There are many kinds of immune responses caused by the tubercle bacilli including circulating antibody, delayed hypersensitivity, increased macrophage activity and granulomatous inflammation (8). The granulomatous reaction of the host is augmented by a T- cell mediated response. Cell wall protein is a major contributor to cell-mediated immune (CMI) response to *M. tuberculosis* organism. Humoral immune (HMI) response involving the production of humoral antibody is not the major immunological defense of tuberculosis infection (8).

2.1 Delayed type hypersensitivity (DTH) response

T-cell mediated response to the tubercle bacillus is a delayed hypersensitivity reaction. After a person becomes infected with mycobacteria, T lymphocytes proliferate and become sensitized. Within weeks these sensitized T cells are circulating in the blood stream. The injection of tuberculin into the skin stimulates the lymphocytes and activates the series of events leading to a DTH response. This response is called “delayed” because the reaction becomes evident only after 24-48 hours. Skin reaction involves vasodilation, edema, and the infiltration of lymphocytes, basophils, monocytes,

and neutrophils at the site of tuberculin antigen injection. Antigen-specific T lymphocytes proliferate and release lymphokines, which mediate the accumulation of these cells at the site. Responses generally peak 48 hours after antigen injection. The area of cellular infiltration or induration reflects DTH activity. Erythema, an acute inflammatory reaction marked by redness at the site of injection can develop in response to a skin-test antigen. DTH response can be detected at 2-10 weeks after initial infection or when the lymphocyte sensitization reaches an adequate level (2). Delayed type hypersensitivity is an accelerated immune inflammatory response that often results in damage to host tissue, especially when local concentrations of tuberculin-like antigens are high (14).

2.2 Cell mediated immune (CMI) response

M.tuberculosis is an intracellular pathogen that can multiply within host macrophages. The immune responses or host defenses mainly depend on T lymphocytes. Antibody production against tuberculosis is only minor importance (6). After infection, the bacilli are phagocytosed by alveoli macrophages and survive within the phagosomes. Innate immune responses directed by macrophages predominate early in infection. The dendritic cells are subsequently recruited. This leads to the cell-mediated immune responses involving CD 4+ and CD 8+ T cells (15). Acquired immune response usually develop after mycobacterial infection 4-6 weeks later and temporally associated with the beginning of delayed hypersensitivity to mycobacterial antigens such as PPD. Successful acquired resistance is mediated by T lymphocytes. Antibodies do not play a protective role against tuberculosis infection although they are present in most of the patients. The mechanisms of immunity against mycobacterial infection is that T lymphocytes activated by specific mycobacterial antigens limit the replication of tubercle bacilli in the infected macrophages. The helper T cell (CD 4+) up-regulate the antigen-specific effector T cells (CD 4+) populations and cytotoxic T cells (CD 8+). CD 4 cells produce cytokines such as gamma interferon (IFN-γ) that activate macrophages and provide them with enhanced mycobactericidal capabilities. The activated macrophages can limit the replication of intracellular *M.tuberculosis* and may kill them. CD 8 cells attack infected macrophages expressing mycobacterial antigens and lyse the

cells, releasing the mycobacteria from their protective condition and exposing them to the activated macrophages (Figure 5).

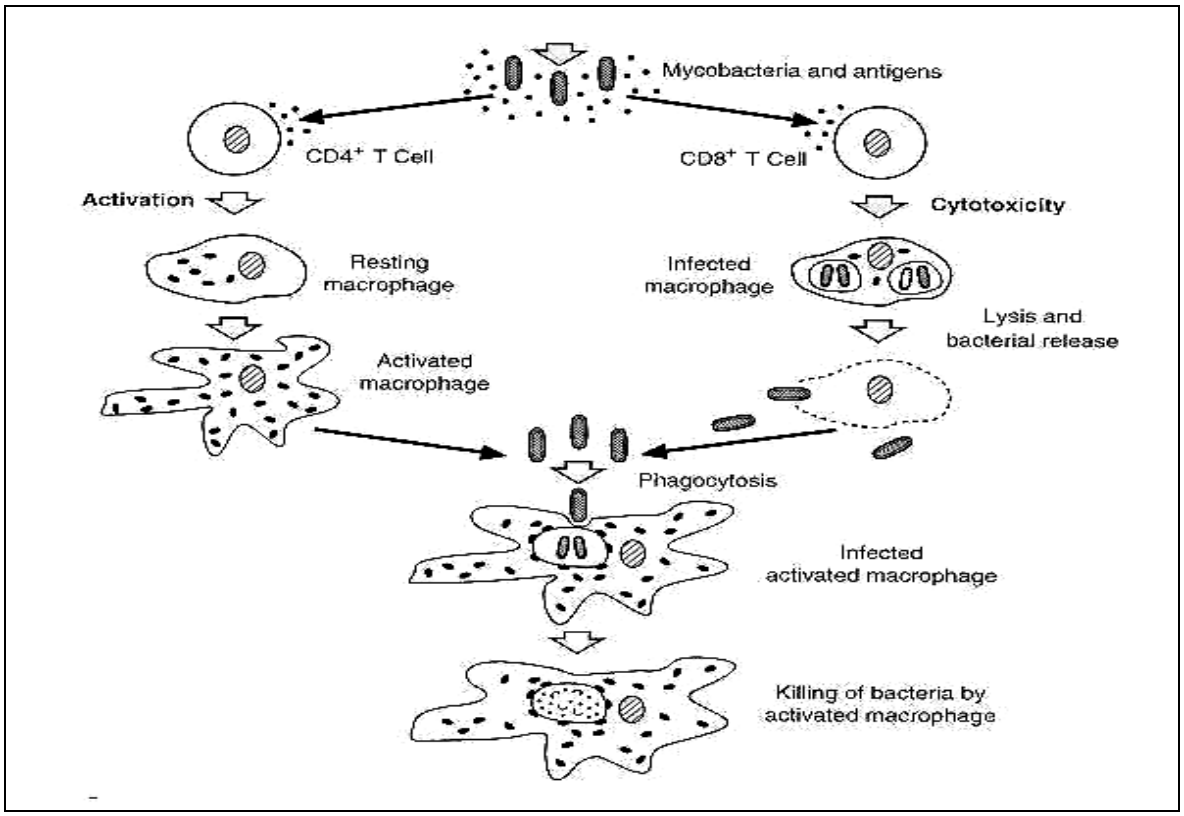

Figure 5 Mechanisms of T-lymphocyte activation or destruction of macrophages following the stimulation of mycobacterial antigens (9)

2.3 The difference between DTH and CMI responses in tuberculosis

In tuberculosis infection, delayed hypersensitivity can be defined as a process that destroys the unactivated macrophages within which tubercle bacilli are multiplying. Cell-mediated immunity can be defined as a process that activates macrophages to kill and digest the bacilli that they ingested. Both responses can stop the multiplication of tubercle bacilli because no bacillary growth occurs in dead macrophages. With these responses, T lymphocytes are locally stimulated by specific antigens of *M.tuberculosis* to produce lymphokines that attract and activate macrophages and additional lymphocytes. This concluded that the main difference between DTH and CMI responses is that the DTH works by killing bacilli-laden unactivated macrophages, but the CMI works by enhancing the macrophages' power to kill the bacilli that they ingested. In

DTH, bacilli are inhibited extracellularly within necrotic sites; but in CMI, bacilli are killed intracellularly within activated macrophages (14).

3. Tuberculin and Purified protein derivative

Tuberculin is a liquid preparation from killed organisms of *Mycobacterium tuberculosis*. The tuberculin skin test is one of the most widely used diagnostic tests and remains the current method for screening tests and epidemiological surveys of human tuberculosis.

3.1 Discovery of tuberculin

It is more than one hundred years that Robert Koch had discovered the bacillus, *Mycobacterium tuberculosis*. After that, he reported in 1891 on the strong response that this tubercle bacilli induced when they were injected into guinea pigs already infected with the same bacillus organisms (10). He did not realize that he had discovered what we use now for detecting tuberculin reactivity or cellular hypersensitivity and become one of the most widely used diagnostic tests. The observation of different responses to tuberculin in patients with and without tuberculosis led to the development of new methods of administering the antigen. By the early 1930, tuberculin skin testing had become a method for screening apparently healthy persons for tuberculosis infection. The material that Koch used was called Old tuberculin. The original method he used was the preparation of tuberculin by growing the tubercle bacilli in a medium of glycerinated meat broth and then killing the organisms with heat in a 100 ^{o}c flowing steam cabinet. The remaining culture medium was concentrated to one-tenth of its original volume over a steam bath. Old tuberculin is no longer in general use because it contains meat broth derivatives (2).

3.2 Tuberculin Purified protein derivative (PPD)

3.2.1 Preparation of PPD and PPD-S

The tuberculin currently used is the purified protein prepared from synthetic-medium. In 1932, Seibert and Monday isolated a low molecular-weight protein by precipitating culture filtrates of tubercle bacilli with trichloracetic acid. In low doses this product was found to be fairly specific for tuberculous infection in both guinea pigs and humans. Efforts to produce a more purified product through precipitation

with ammonium sulfate resulted the PPD. It has been studied by a variety of techniques and has been shown to contain a number of antigenic components, most of which are low and medium-weight proteins. In 1941, Seibert showed that the active principle in tuberculin was protein and this led the way to the preparation of a purified protein derivative (PPD) of tuberculin, which is the form now most commonly used. Tuberculin PPD is prepared from the water-soluble fractions obtained by steaming and subsequently filtering cultures of the tubercle bacillus grown in a liquid synthetic medium. The active fraction in the filtrate is isolated and purified by repeated precipitation and washing. The end product either as a concentrated solution or a dried powder is carefully assayed against a standard preparation in laboratory animals, and from time to time the laboratory results are verified by comparative tests in humans. The need for a standardized and reliable preparation of tuberculin was recognized by Florence Seibert who worked at the Henry Phipps Institute of the United States. The standard for all PPD preparations is Tuberculin PPD lot number 49608, or PPD-S. Aliquots of the lot prepared in 1941 by Seibert and Glenn were sent to the Division of Biologics Standards at the National Institute of Health for use as the U.S. reference standard; to various commercial and private concerns for research studies; and to the State Serum Institute in Copenhagen, Denmark. In 1952, PPD-S was adopted as the International Standard for Purified Protein Derivative of Mammalian Tuberculin by the World Health Organization (2).

3.2.2 Use of diluted tuberculin PPD

The tuberculin reagent for use in skin test has to be diluted from the concentrated PPD solution to the strength that is equivalent to 5 IU dose of Standard PPD or PPD-S. The strength of 1 IU is defined as the biological activity of tuberculin contained in 0.000028 mg of PPD-S. In the U.S. and Canada, the potency of PPD preparations is expressed in TU rather than IU (2). The conventional use of 5 test units (TU) is the bioassayable skin test activity contained in 0.0001 mg of PPD-S. In 1942, Furcolow et al. also recommended substitution of a single 5 TU (“intermediate-strength”) injection, on the basis of the fact that 5 TU caused reactions in 99.6% of patients with active tuberculosis. At present, all commercially produced, stabilized, PPDs were required to be bioassayed and shown equipotent to 5 TU of PPD-S before marketing. Since then, master lots of PPD products marketed in the United States have

been standardized by bioassay in both tuberculin sensitized guinea pigs and skin test-positive humans (17).

3.2.3 Liquid tuberculin PPD and its shelf life

Tuberculin becomes very unstable when highly diluted so that it should be used only if it has been recently prepared. The potency of tuberculin always varies that is difficult to control and predict the stability. One major factor that indicates the instability of tuberculin dilution is the loss of its biological activity (5).

The dilutions of PPD in room temperature quickly lose their activity in 24 hours however there has been some experiments indicating that the diluted purified tuberculin maintains its potency up to 6 months (3,18,19). Shelf life of tuberculin PPD is actually concerned with the remaining activity after processing and storage. There is no question that the activity of tuberculin accompanies protein. Degradation of protein may result in loss of biological activity. Although liquid formulation is preferred in terms of cost, ease of packaging and convenience to the end user, many proteins are not stable enough in liquid solutions to meet shelf life requirements. Water is an active participant in many chemical degradation processes in proteins. In addition, liquid formulation often lack robustness with respect to temperature excursions, and must be carefully protected against accidental heating or freezing during shipping and storage. Lyophilization is the resolution when adequate stability and suitable shelf life cannot be obtained in a liquid formulation (20).

4. Assessment of tuberculin activity

4.1 Assay for biological activity

The assay for biological activity or potency of tuberculin is supplemented by estimation of the protein content because chemical analysis has not given an accurate measurement of the biological activity. In 1951, Rich observed that tuberculo-proteins exert no appreciable toxic effect on the normal body but are highly toxic for the tuberculous, hypersensitive body (21). He defined tuberculin as any material, other than living tubercle bacilli, that contains tuberculo-protein or degeneration products of tuberculo-protein, and which is active in eliciting a hypersensitivity reaction in the sensitized body. When tuberculin is administered intradermally, a hypersensitivity

reaction manifesting as induration will appear in the subject sensitized with mycobacteria. A positive reaction is an indication that the patient has had, at some time a tuberculous infection. An infected individual probably retains hypersensitivity to tuberculin as long as viable bacilli and memory cells remain within the body. The size and intensity of the reaction depends on the amount of tuberculin or volume of material injected and on the degree of hypersensitivity of the subject (21).

The biological activity of tuberculin in a subject infected with tubercle bacilli are due to an unknown number of different substances, which are present in varying concentrations in different tuberculin preparations. The response to tuberculin tests with various products vary with the biological material, the test technique, the tuberculin dose, etc. The estimation of tuberculin potency will depend on how the assessment is made. Based on the knowledge that there is no chemical method available for satisfactory accuracy of the assessment of the tuberculin biological potency, the potency of tuberculin products must therefore be assayed by means of biological material. In most laboratories this biological assessment is performed in guinea-pigs, which are sensitized either with virulent tubercle bacilli, with BCG or with heat-killed tubercle bacilli suspended in mineral oil. The potency values thus obtained are in general considered to be valid also for humans and for any technique applied (22). The BCG-induced non-specific immunity has been studied and noted that BCG has a stimulatory effect on the immune response to heterologous antigens. (23). On the other point of view, tuberculin PPD contains antigens that are shared among pathogenic mycobacteria, environmental mycobacteria, and the vaccine strain *Mycobacterium bovis*, BCG (24, 25). In all cases, potencies of tuberculin products have been measured by intradermal testing in BCG-vaccinated guinea pigs (5).

4.2 Assay for tuberculin Interferon-gamma (IFN-γ)

The assay for tuberculin Interferon-gamma (IFN-γ) levels is based on the knowledge about host defenses against tuberculosis that depends on measuring specific T-cell reactivity. The DTH response in tuberculin skin test, as an *in vivo* test, measured by the skin reaction has been shown to be dependent on the production of cytokines, including IFN-γ**,** at the site of tuberculin injection. An *in vitro*, human IFN-γ immunoassay has recently been developed and used for human tuberculosis in comparison with tuberculin skin testing. IFN-γ responsiveness from *in vitro* test was

correlated with *in vivo* tuberculin reactivity. The Enzyme Immunoassay for IFN-γ was shown to be highly sensitive, detecting less than 0.5 IU of recombinant human IFN-γ per ml within a linear detection range of 0.5 to 150 IU/ml. This method was highly reproducible and specific for native IFN-γ (6).

IFN-γ assay works on the same principle as Mantoux test but being an *in vitro* test (26). The principle of IFN-γ assay is that individuals primed *in vivo* with antigen have lymphocyte in their blood that maintained an immunological memory for the priming antigen (27). Addition of antigen to blood taken from primed individuals results in rapid re-stimulation of antigen-specific memory T cells and the secretion of cytokine Interferon-gamma (Figure 6) which is used as a specific marker for a CMI response mounted to the antigen (28). Stimulation of memory T cells in whole blood with specific antigen or mitogen and subsequent quantification of IFN-γ by a rapid, single-step ELISA forms the basis of this assay (6).

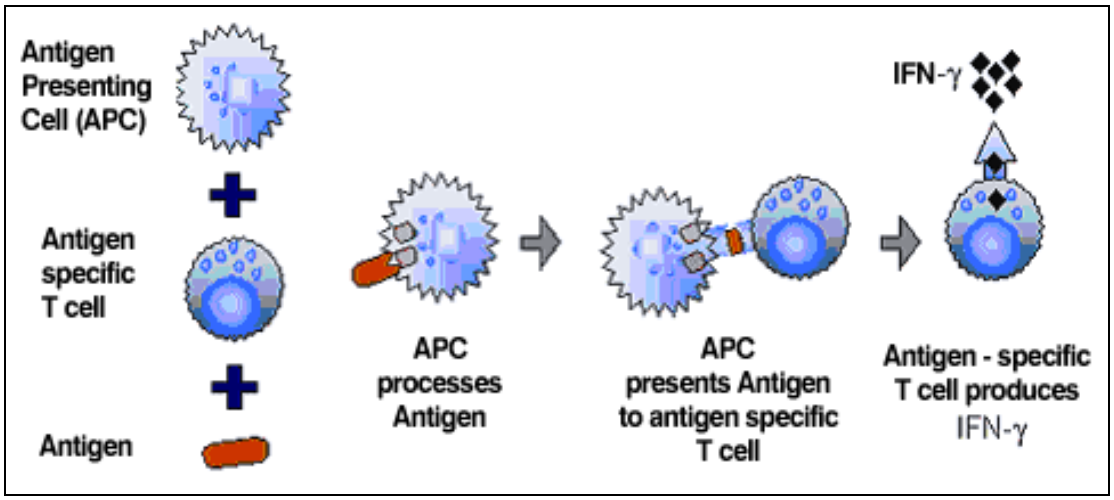

Figure 6 Secretion of IFN-γ after stimulated the Ag-specific T cell with antigen (29)

5. Method of tuberculin test in humans

Tuberculin skin test has been used for detection of tuberculo-protein hypersensitivity. The tuberculin reaction is equivalent to the Koch's phenomenon (30). Koch emphasized the evidence that if a normal guinea pig is inoculated with a pure

culture of tubercle bacillus, the wound closes rapidly and heals in the first few days. After 10-14 days, a firm nodule appears at the site of injection which opens forming an ulcer. This typically persists until the animal dies. However, if the same culture of tubercle bacillus is injected in a tuberculous guinea pig (an animal infected 4-6 weeks earlier) it yields a different result. A peculiar change occurs at the inoculation site on the first or second day: the tissue surrounding the site of injection becomes indurated and dark-coloured and in the next few days it becomes necrotic. It finally sloughs leaving a shallow ulcer which usually heals quickly and permanently without infection of the local lymphnodes. Koch also established that killed bacilli or even tuberculin could substitute bacilli and result in the same local tissue reaction (30). The tuberculin skin test used in humans now can be differentiated by the techniques of administrations and the interpretations of the results. Tuberculin purified protein derivative is administered by syringe in the Mantoux test or by means of a multiple puncture gun in the Heaf test. Development of significant induration generally indicates hypersensitivity to tuberculo-protein; vaccination is not indicated but investigation for active tuberculosis may be necessary (31).

5.1 Mantoux test

The Mantoux test, 0.1 ml containing 5 IU of PPD solution is injected intradermally on the volar surface of the upper third of the forearm (as shown in Figure 4). The result is read 48 to 72 hours later as the area of induration, with at least 5 mm in diameter is the threshold for indication of a positive reaction (8).

5.2 Heaf test or Tine test

The Heaf test or Tine test, a drop of undiluted PPD (100,000 IU/ml) was spread with a glass stick on the forearm, after which an apparatus with six needles is used to inject about 50 IU into the epidermis. The results are read 5 to 7 days later and recorded as grade 1 to 4, depending on the appearance of the papules at the puncture sites. Grades 2 to 4 are interpreted as positive. The Heaf test uses quite a lot of tuberculin and does not give quantitative results (8).

6. Lyopilization and formulation

6.1 Lyophilization strategy

To overcome the instability problem, proteins have to be made into solid forms to achieve an acceptable shelf life. The lyophilization process is needed because it is commonly used to prepare protein products for long-term storage when the stability of aqueous formulation is insufficient. Although preparation of lyophilized proteins is an expensive, time-consuming, and typically batch process, the lyophilized formulation of protein offers the advantages over liquid formulation because the air-water interface is removed so that freeze-dried proteins are less susceptible to mechanical stresses during shipping and handling (20). Lyophilization stabilizes the formulation by slowing the kinetic clock of the degradation process. It alters the clock by removing the solvent component or components to levels that no longer support chemical reactions or biological growth (32).

6.2 Formulation and excipients

The development of the formulation is beneficial to a lyophilized product. A formulation can be defined as a liquid medium in which one or more active components (chemical or biological) remain in a stable environment and maintain specified potency limits for some period. The formulation consists of three basic components; active ingredient, excipients and solvent system. The active ingredient in the pharmaceutical industry is generally defined by its potency and in the diagnostic industry, by its reactivity. Depending on the methods of production, there may be variation in the composition of the active component from batch to batch (32). A protein formulation requires some kinds of excipients as the stabilizers to protect protein stability both during freezing and drying process because the lyophilization process can denature proteins to various degrees. Even after successful lyophilization, the long-term storage stability of proteins may still be very limited, especially at high storage temperature. In general cases, protein stability in solid state has been shown to be equal to, or even worse than, that in liquid state, depending on the storage temperature and formulation composition (33)

Stability of protein in the product also depends on the process of preparation and the nature of excipients added in the solution. The powerful additives selected for

formulations are not only stabilize, but also enhance effect of protein activity. The stresses associated with freezing and drying can often cause irreversible damage to the protein, seen as denaturation upon dehydration and loss of biological efficacy so that addition of protective excipients is also required. In the typical approach to protecting proteins against the stresses during freezing and drying in the lyophilization process, excipients for cryo-protection and lyo-protection such as sugar, amino acid and polymer should be added to the lyophilized formulations as stabilizers (34).

Many sugars or polyols are frequently used as nonspecific protein stabilizers in solution and during-thawing and freeze-drying. They have been used as both effective cryoprotectants and remarkable lyoprotectants. The level of stabilization afforded by sugars or polyols generally depends on their concentrations. Since freezing is part of the freeze-drying process, high concentrations of sugars or polyols are often necessary for lyoprotection. The level of protein protection afforded by different sugars or polyols can be either similar or significantly different, depending on the formulation composition, concentration and physical properties of the stabilizer, and its compatibility with the protein. Polymers have also been used to stabilize proteins in solution and during freeze-thawing and freeze-drying. Serum albumin was one of the favorable polymers used in the history of protein drug development. It has been used both as cryoprotectant and lyoprotectant. In addition to albumin, other polymers also have been used. The level of protein stabilization afforded by the polymers depends on structure and concentration of both the polymer and the protein. One of the properties of polymer attributed in stabilization of proteins is that it can increase solution viscosity and limit the protein structural movement. In recent years, additional properties of polymers have been implicated in stabilizing proteins during freeze-thawing and freeze-drying (33).

Excipients of different properties can serve several functions; for examples:

Haemaccel is a degraded gelatin (polygeline) that has the properties as polymer. It can increase solution viscosity and limit protein structural movement (33). Haemaccel is pharmacologically inert, manufactured from gelatin derived from bovine material. It is a clear, sterile, pyrogen free and ready for use solution which contains no preservatives. In addition, Haemaccel can be used as a vehicle for various medicines (35)

Trehalose is a disaccharide, two simple sugars in one molecule. In trehalose, the two sugars are both glucose. It has a very low hygroscopicity, so it is less attractive to moisture and stays free-flowing and dry. Uses of trehalose are for foods as sweetener, a stabilizer and thickener and a flavor enhancer. It is also used as a cryopreservation additive, where it protects cells from the effects of freezing and drying (36).

Mannitol is an alcohol and a sugar, or a polyol. Many sugars or polyol are frequently used nonspecific protein stabilizers in solution during freeze thawing and freeze drying. They have been used as both effective cryoprotectants and remarkable lyoprotectants (33). Mannitol has a property of limited water solubility and remains powdery and granular on long storage. It is widely used in medicine and as a dietary supplement (37).

Sucrose is the chemical name of table sugar. It is a disaccharide, normally used for protein stabilization (33). It is generally extracted from sugar cane or sugar beet and then purified and crystallized. Pure sucrose is the most common sweetener in the modern, industrialized world (38).

6.3 Lyophilization process

The principle of lyophilization is to dry (remove water or solvent material through the process of sublimation), thereby maintaining chemical characteristics and increasing shelf life (39).

The lyophilization process of a protein consists of two major steps: Pre-freezing of the solution, and drying of the frozen solid under vacuum. The drying step is further divided into two phases: primary and secondary drying.

Pre-freezing: The products are loaded and frozen until all the water becomes ice.

Primary drying: The ice turns directly to vapor under vacuum by sublimation (the transition of a substance from the solid to the vapor state, without first passing through an intermediate liquid phase).

Secondary drying: The remaining water, bound to molecules bonding to the solute, is removed from the product. If the active ingredient were a protein, then

perhaps over-drying could result in a change in the configuration of the protein and a subsequent loss in potency of the final product (40).

7. Analysis of stability

Method for determining the stability of biological products is based on a knowledge about protein's stability because the vaccines, toxoids or antigens are made up of proteins which undergo changes on exposure to heat. Most of biological materials consist of large and complex molecules and it is a characteristic of such molecules that they are susceptible to thermally-induced conformational changes which significantly reduce their activity (41). The estimation of loss of potency during various periods of storage at different temperatures involves the method of accelerated degradation test (ADT). The samples are subjected to a range of elevated temperatures at which significant and readily detectable degradation is induced in relatively short time (42). Test of the stability of biological product can be done by comparison of the activity of product before and after storage in terms of the animal-response stability test that requires the bioassay (43).

CHAPTER III
MATERIALS AND METHODS

1. PPD and excipients

Concentrate of tuberculin PPD was purchased from Chiron S.p.A (Seina), Italy, Lot No.57/58 ; 24,180 μg of total protein content per container. The strength of tuberculin PPD is expressed as 487,500 units per container. The powder bulk was reconstituted precisely by 5 ml of Phosphate buffered solution , pH 7.38

Sucrose (Merck, 1.07651, E Merck, Germany), Mannitol (Merck, 5980, E Merck, Germany), Trehalose (Merck, 1.08216.0100, E Merck, Germany) and Haemaccel 3.5% solution (Polygeline, Hoechst Marion Roussel Deutschland, Germany) were used as excipients in concentrations as described in Part of preparation of diluted tuberculin PPD.

2. Animals and test kits for analysis

Guinea pigs (White, Albino) weighing 400-450 g were sensitized with BCG vaccine (Lot No. 653, manufactured by QSMI, Thai Red Cross) intradermally on the abdomens. The vaccine contains 0.05 mg of BCG per dose. The injections of tuberculin PPD solutions under test were done in the 30 immune guinea pigs per group of experiment, at 6 weeks after immunization with vaccine in order to ensure adequate immunological reactivity.

The QuantiFERON-TB test manufactured by Cellestis Limited, Victoria, Australia and approved by the U.S. Food and Drug administration (44) was used for Tuberculin Interferon-γ Assay. The samples of heparinized venous blood were collected from 5 tuberculin skin test-positive individuals responded to the human tuberculin PPD antigen in Mantoux tests with the diameter of reaction sizes of ≥5 mm.

3. Design of the formulations

In this experiment, sugars or disaccharides (Sucrose, Mannitol, Trehalose) and gelatin (Haemaccel or polygeline) were chosen as excipients for comparison study. Sugars can inhibit molecule unfolding of protein during freeze drying and gelatin can increase solution viscosity and limit the protein structural movement. The level of protein stabilization afforded by excipients generally depends on their concentrations. The final concentration of sugars should be between 2% to 7% which is the suitable range. Gelatin should be used in final concentration of not more than 0.5% (33). These stabilizing agents will be added to the formulations as cryo-and lyo-protectants of proteins in the lyophilization process. The concentrations of the solutions prior to lyophilization are 5.0% Sucrose (S formula), 6.5% Mannitol (M formula), 2.5% Trehalose (T formula) and 0.3% Haemaccel (H formula).

4. Lyophilization

To achieve the satisfactory characteristics and qualities of lyophilzed product, an optimized program for freeze drying was selected to run with the parameters concerning cooling rate, heating rate, pressure limit and total time of cycle. This is to obtain a consistent, stable and acceptable lyophilized product.

Lyophilization cycle for tuberculin PPD samples was designed for the steps as the followings: (the profile was shown in Figure 7)

1. Loading and Pre-freezing

Loading temperature	+ 20 ^{o}C
Freezing temperature	– 35 ^{o}C (after shelf cooling 1.5 hrs)
Cooling rate	0.6 ^{o}C / min
Holding time	4.5 hrs

2. Primary drying

Ramp	0 ^{o}C in 20 hrs
Heating rate	0.03 ^{o}C /min
Pressure	< 5.0x10^{-2} mbar
Holding time	4 hrs

3. Secondary drying

Ramp	+20 ^{o}C in 5 hrs
Heating rate	0.06 ^{o}C /min
Pressure	< 5.0x10^{-2} mbar
Holding time	13 hrs

Total cycle time was 48 hrs

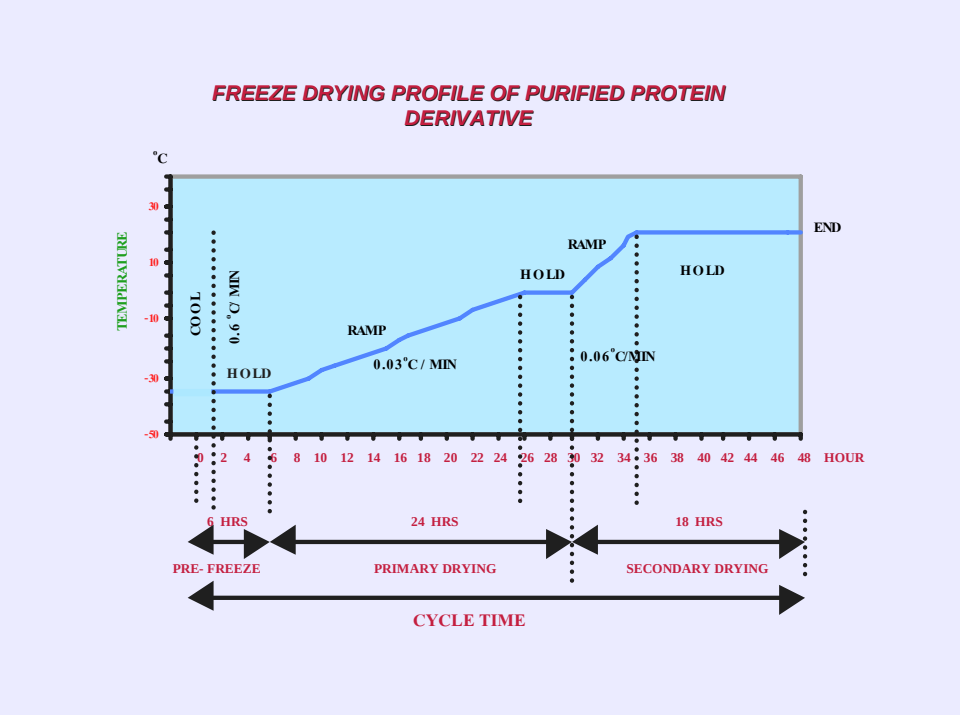

Figure 7 The profile of lyophilization process of Tuberculin PPD

5. Preparation of diluted tuberculin PPD

Concentrate solution of tuberculin PPD bulk (97,500 units/ml) was diluted to 100 units/ml by using phosphate buffered solution, pH 7.38 containing 5 ppm Tween 80 and 0.3 % Phenol, as diluent. The lyophilized formulations were prepared in double strength of the liquid formulation or 200 units/ml with the addition of each experimental excipient as designed. There were 4 formulations of 5.0% Sucrose (S formula), 6.5% Mannitol (M formula), 2.5% Trehalose (T formula) and 0.3%

Haemaccel (H formula) prepared pior to lyophilization. Aliquots of 0.5 ml of each formula were filled into the first group of 2 ml vials and lyophilized for 48 hrs in the lyophilizer, model FD 20 of Heto Lab equipment company, Denmark. To preserve the dried products with vacuum condition inside the vials, the rubber stoppers were closed under high vacuum of the lyophilizer chamber and then they were forwarded to be sealed tightly with aluminium caps. When the test for analysis was performed, reconstitution of a lyophilized sample was done by adding 1.0 ml of water for injection to yield the same concentration (100 units/ml) as liquid samples.

6. Preparation of controls

6.1 Control of lyophilized tuberculin PPD without excipient

Aliquots of 0.5 ml of diluted tuberculin PPD in the absence of excipient were filled into the second group of 2 ml vials and freeze dried in the same cycle of the lyophilized samples (as described in 4). These were identified as CD formula.

6.2 Control of lyophilized excipients without tuberculin PPD

Bulk of the same diluent (Phosphate buffered solution, pH 7.38 containing 5 ppm Tween 80 and 0.3 % Phenol) in the absence of tuberculin PPD was separated and the solutions were prepared by adding 5.0% Sucrose (ES formula), 6.5% Mannitol (EM formula), 2.5% Trehalose (ET formula) and 0.3% Haemaccel (EH formula) into each containner. Aliquots of 0.5 mL of each formula were filled into the third group of 2 ml vials and lyophilized in the same cycle of lyophilized samples. This control was designed for detection of allergic effects of excipients used that may falsify the reactions occurred in the biological assay.

6.3 Control of liquid tuberculin PPD without excipient

Aliquots of 1.0 ml of the liquid formulation (100 units/ml) as described in the part of preparation of diluted tuberculin PPD were filled into the fourth group of 2 ml vials and labeled as CL formula. These samples were kept cool immediately after preparation at 4°c.

7. General tests

Residual moisture content of lyophilized products of tuberculin PPD were determined by Karl-Fischer titration (Orion, model AF 8, Orion, USA). pH of tuberculin PPD solutions were measured by a pH meter (Orion, model 520A,Orion, USA)

8. Analysis of the experimental products

8.1 Biological assay for tuberculin activity

The biological test for tuberculin activity of each formulation was carried out by detecting DTH responses to PPD in skin tests of 30 sensitized guinea pigs that had been vaccinated with BCG vaccine for 6 weeks as previously described. The tests were done by injecting intradermally into the guinea pigs with 0.1 ml of the solutions from the formulations of CL, CD, S, M, T, H and followed by 0.1 ml of the negative control from ES, EM, ET, EH formulations. Each guinea pig was injected with 10 samples distributed at random among various sites on the abdomen skin as shown in Figure 8. Reading of the reactions caused by tuberculin activity was done 24 hours after PPD injection. Longitudinal and transverse measurement of diameters of indurations at the injection sites were measured and recorded in mm. A reaction from readings (n=30) is the average figure of 2 measurements at 1 site.

Statistical analysis was done by paired t-test on the data of reaction sizes of lyophilized formulations (CD, S, M, T, H) and liquid formulation (CL) which was assumed to be gold standard for comparisons of paired two sample for means. The acceptable formulation for further stability study must show no significant difference (P value >0.05).

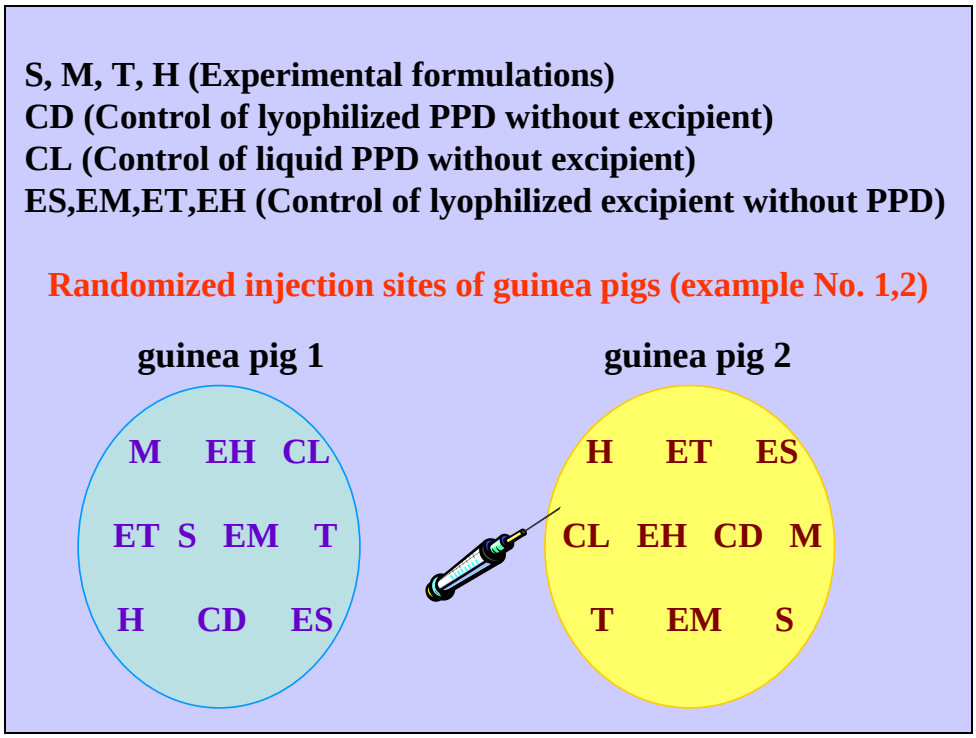

Figure 8 Injections of test samples distributed at random among various sites on the abdomen skin of guinea pigs

8.2 Tuberculin Interferon-gamma (IFN-γ)Assay

The tuberculin activities remained in the products of liquid formulation (CL) and lyophilized formulations (CD, S, M ,T ,H) were expressed in terms of the percentage of tuberculin responses (% responses to *M.tuberculosis* antigens or tuberculin antigens in each of these formulas). This method was used for detection of CMI responses by measuring IFN-γ in plasma harvested from whole blood incubated with tuberculin PPD antigens, mitogen (phytohemagglutinin) as positive control (Pc) and phosphate buffer saline solution (PBS) as negative control or Nil control (Nc). The QuantiFERON-TB laboratory test was done by following the instructions of manufacturer (Cellestis Limited, Victoria, Australia), except only the solutions of CL, S, M, T, H were used as test antigens instead of PPD antigen from the manufacturer. Method of this test (as shown in Figure 9) is that the samples of whole blood from tuberculin skin test positive individual were gently mixed by inverting the tubes and 5 samples were tested in duplicate (n=10). Blood sample of 1.0 ml was transferred into each well of a microplate. Each tuberculin PPD product as our test antigen was

prepared to 5 μg/ml and 100 μl (0.5 μg) was used for stimulation of 1.0 ml blood. The antigens of 100 μl of tuberculin PPD including 3 drops of a negative control (PBS) and 3 drops of a positive control (mitogen) were added to stimulate each individual aliquot of blood. The plate was shaken for 1 min and incubated for 20 hours at 37°c. After incubation, the supernatants were collected and 50 μl of each supernatant was quantified for the amount of IFN-γ by enzyme-linked immunosorbent assay (ELISA) using the QuantiFERON-TB test kit according to the manufacturer's instruction manual. Human recombinant IFN-γ standards, which have been assayed by the manufacturer against a reference IFN-γ preparation (NIH Ref:Gxg01-902-535) was measured by ELISA and used for the standard curve. A linear curve of known IFN-γ standards was plotted, and the results of test samples were read in terms of International Units (IU) relative to the standard preparation. The IFN-γ produced in response to test tuberculin PPD antigen in excess of negative control (Ag-Nc) and the IFN-γ produced in response to mitogen in excess of negative control (Pc-Nc) were calculated for % Response to tuberculin PPD antigen. Formula for calculation is indicated as follows:

$$\% \text{ Tuberculin Ag Response} = \frac{\text{Ag - Nc}}{\text{Pc - Nc}} \times 100$$

The IFN-γ levels in the samples were determined for evaluation of remaining tuberculin activity in each formulation. The average level of IFN-γ in response to tuberculin PPD antigens were reported in terms of % responses obtained from PPD antigens of the experimental formulation.

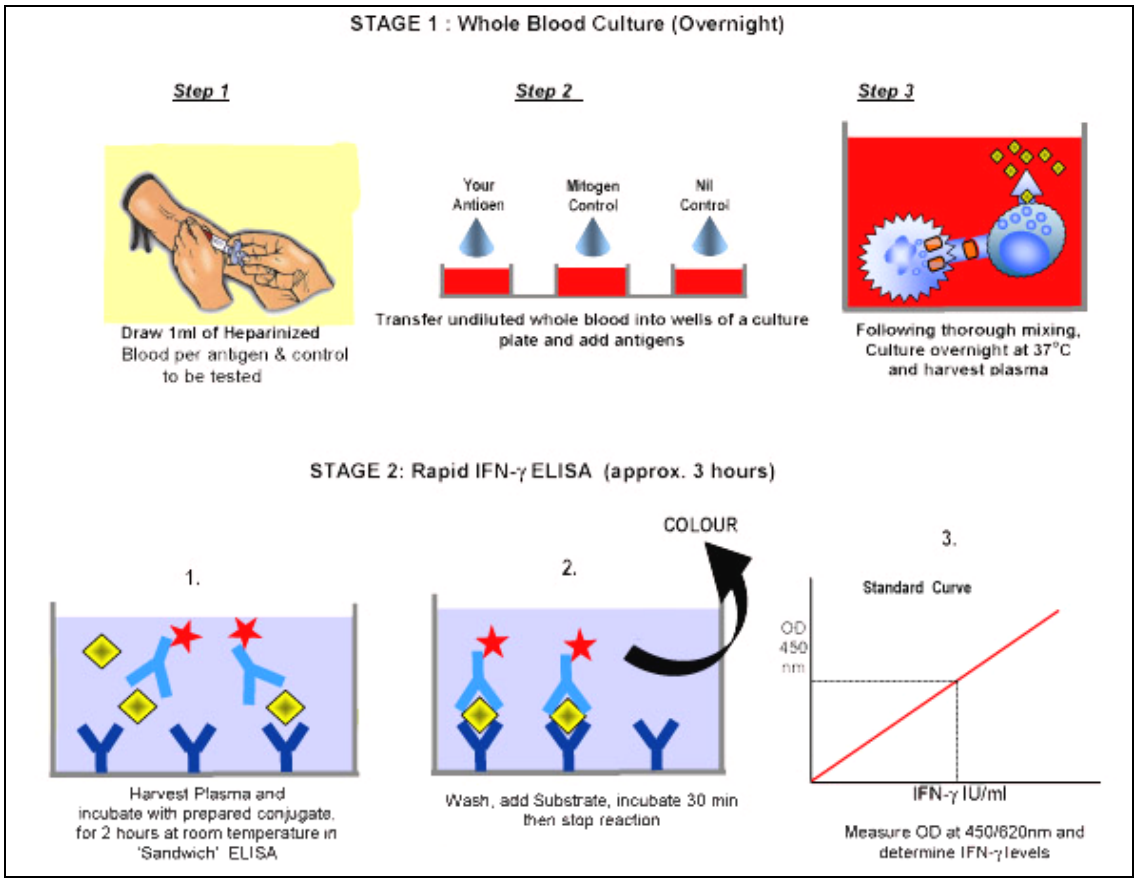

Figure 9 A summary of Interferon-gamma Test Procedure, using whole blood culture and ELISA method (45)

8.3 Stability study

Accelerated degradation test was performed to monitor the remaining tuberculin activities of lyophilized formulations (M, T, H) that have been accepted on the results of bioassay and the original liquid formulation (CL). The test samples were subjected to elevated temperatures of the two incubators at 37°c and 56°c and normal storage temperature of the refrigerator at 4°c for various periods of 3, 6, 9 and 12 months. Biological assay for the determination of degradation at each interval of storage was done by parallel DTH skin test in each animal of a group of 30 BCG vaccinated guinea pigs. Each guinea pig was injected intradermally with 12 test samples distributed at random among various sites on the abdomen skin. Reading of the reactions was done after 24 hours in the same manner as performed in the first biological assay. A mean reaction was the average of reactions from 30 injections of each sample. Percentage of remaining activity of each formulation after storage in

various conditions was calculated by comparison with the initial activity of liquid formulation before storage or at period of 0 month. The demonstration of stability study was shown by curves plotted between the remaining activities and the storage times.

CHAPTER IV
RESULTS

The design of formulations and lyophilization process of tuberculin PPD was completed by selecting Sucrose (S), Mannitol (M), Trehalose (T) and Haemaccel (H) as excipients and the concentrations in the solutions prior to lyophilization were made as described in the part of materials and methods. After lyophilization, all the products of S, M, T and H formulations have shown the satisfactorily physical characteristics and the general tests for residual moisture content and pH measurement of each formulation were performed before the analysis.

1. Residual moisture contents and pH

The residual moisture content of each lyophilized product results within the limit (S = 3.6%, M = 1.2%, T = 6.7% and H = 8.5%). In general, moisture content of lyophilized protein product was acceptable when it was below 10% (33).

The pH of each formulation after reconstitution with PBS results within the normal range (S = 7.34, M = 7.35, T = 7.27 and H = 7.35). The pH required for tuberculin solution was ranging between 6.5-7.5 (1).

2. Biological assay for tuberculin activity

The result of study on tuberculin activities of lyophilized formulations (S, M, T, H), liquid formulation (CL) and control (CD) showed the different levels of DTH responses in 30 immune guinea pigs. Reactions due to DTH responses were measured at 24 hours after injections of tuberculin PPD antigens of the different formulations into the guinea pigs (Figure 10). Mean size of reactions in mm and the percentage of remaining activities were shown in Table 1. The percentage of tuberculin activities of lyophilized formulations resulted by comparison of the activities of lyophilized formulations with the initial activity of liquid formulation. They varied according to the efficacies of excipients used for stabilization of proteins during processing and

lyophilization. The H-formula showed the highest activity (100% remaining activity) among those experimental excipients.

The results of statistical analysis presented in Table 2 showed that there were significant difference between CD and CL and between S and CL (P value <0.05). No difference was found from M, T, and H formulas when compared to CL. In addition, the negative control of ES, EM, ET and EH formulas which were absent of tuberculin PPD did not reveal any reactions or side effects at the site of injections.

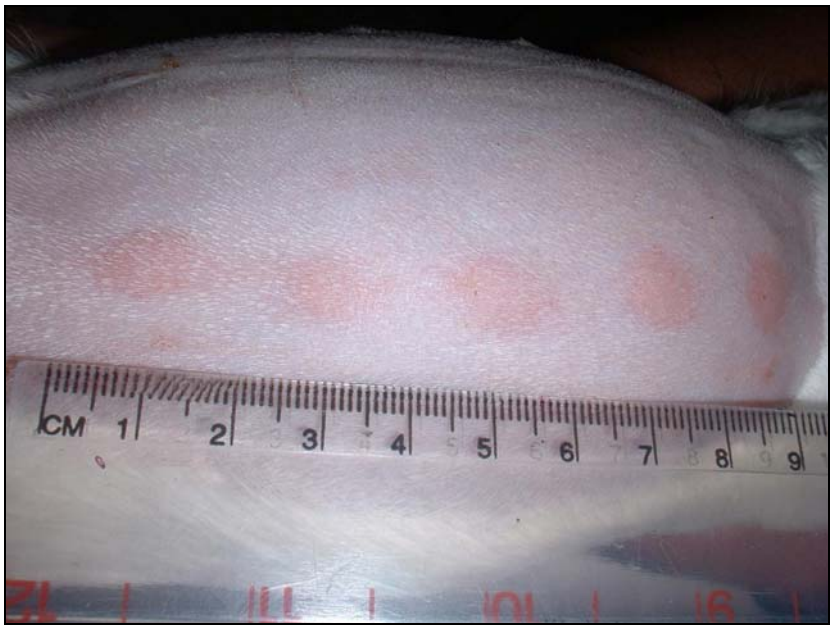

Figure 10 Different levels of DTH responses caused by injections of the test tuberculin PPD samples in the immune guinea pig

Table 1 Comparison between efficacies of lyophilized formulations and liquid formulation by DTH responses in guinea pigs

Formulation	Mean size of reaction (mm.)	Remaining activity (%)
CL (Liquid)	7.8	100
CD (Lyophilized)	6.3	83
S (Sucrose)	4.7	60
M (Mannitol)	7.0	90
T (Trehalose)	6.9	89
H (Haemaccel)	7.8	100
ES, EM, ET, EH	no reaction	-

(Each mean is the average of reactions from 30 tests)

CL: Liquid

CD/ S/ M/ T/ H: Lyophilized (CD: Control of lyophilized without excipient)

ES/ EM/ ET/ EH: Lyophilized without tuberculin PPD

Table 2 Statistical analysis of tuberculin activities of DTH response in guinea pigs by paired t-test as expressed by mean size of reaction of each formulation compared to liquid formulation

	Liquid	Lyophilized formulation				
	CL (Liquid)	CD (Control)	S (Sucrose)	M (Mannitol)	T (Trehalose)	H (Haemaccel)
Mean (mm)	7.80	6.25	4.72	7.02	6.88	7.83
Variance within sets	3.27	8.81	13.74	7.82	6.46	7.14
Standard deviation	1.81	2.97	3.71	2.80	2.54	2.67
t Stat		2.42	4.71	1.71	1.88	-0.08
P (T ≤ t) two-tail		0.02	0.00006	0.17	0.07	0.94
t Critical two-tail		2.05	2.05	2.05	2.05	2.05

3. Tuberculin Interferon-gamma assay

Tuberculin activities of lyophilized formulations (S, M, T, H) and controls (CL, CD) were analysed by measurement of IFN-γ quantities. Based on the Standard curve with the Correlation coefficient (r)=0.999 (as shown in Figure 11), IFN-γ secretion (IU/ml) against a panel of the tuberculin PPD antigens contained in these lyophilized formulations are shown in Table 3. For comparisons of IFN-γ levels among the tests on tuberculin PPD formulations, percentage of responses to tuberculin antigens were calculated from the values of IU/ml. The results presented in Table 4 demonstrate that H formula is superior in its ability to induce IFN-γ secretion to the other experimental formulas. It indicates the stronger IFN-γ response (10.40%) than the responses from CL, CD, S, M and T formulas.

The results of tuberculin activities of the experimental formulations from the *in vivo* and the *in vitro* analyses are summarized by Table 5.

IFN-γ concentration (IU/ml)	**Absorbance** 450 nm(OD)
0	0.022
15.8	0.305
64.1	1.280
123.0	2.324

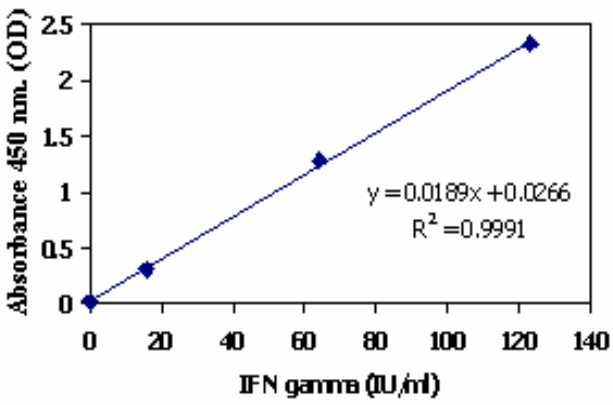

Figure 11 Standard curve of the absorbance against the known IFN-γ concentration: The linearity of standard curves, expressed as correlation coefficient (r = 0.99)

Table 3 IFN-γ secretions from blood samples in responses to tuberculin antigens contained in the experimental formulations

*Blood Sample	IFN-γ secretion quantity as determined from the standard curve (Fig.11)															
	CL (Liquid)		CD (Control)		S (Sucrose)		M (Mannitol)		T (Trehalose)		H (Haemaccel)		Mitogen		PBS	
	OD	IU/ml	OD	IU/ml	OD	IU/ml	OD	IU/ml	OD	IU/ml	OD	IU/ml	OD	IU/ml	OD	IU/ml
1	0.191	8.698	0.024	-0.138	0.032	0.286	0.029	0.127	0.057	1.608	0.364	17.852	2.571	134.624	0.063	1.926
2	0.177	7.958	0.028	0.074	0.039	0.656	0.034	0.392	0.045	0.974	0.346	16.899	2.494	130.550	0.025	-0.085
3	0.061	1.820	0.057	1.608	0.068	2.190	0.070	2.296	0.061	1.820	0.263	12.508	1.858	96.899	0.024	-0.138
4	0.060	1.767	0.038	0.603	0.046	1.026	0.045	0.974	0.064	1.979	0.268	12.772	1.781	92.825	0.032	0.286
5	1.105	4.148	0.055	1.503	0.044	0.921	0.058	1.661	0.083	2.984	0.246	11.608	2.020	105.471	0.018	-0.455
6	1.120	4.942	0.042	0.815	0.040	0.709	0.030	1.180	0.069	2.243	0.222	10.339	2.125	111.026	0.020	-0.349
7	0.084	3.037	0.020	-0.349	0.089	3.302	0.055	1.503	0.065	2.032	0.202	9.280	1.985	103.619	0.032	0.286
8	0.090	3.354	0.026	-0.032	0.075	2.561	0.041	0.762	0.052	1.344	0.109	4.360	2.099	109.651	0.011	-0.825
9	0.055	1.503	0.047	1.079	0.053	1.397	0.038	0.603	0.059	1.714	0.198	9.069	2.218	115.947	0.029	0.127
10	0.062	1.873	0.038	0.603	0.048	1.132	0.050	1.238	0.040	0.709	0.205	9.439	2.189	114.413	0.037	0.550

Mitogen = Positive control PBS = Negative control

* Blood samples were taken from 5 tuberculin skin test positive individual and tested in duplicate

Table 4 Percentages of IFN-γ responses to tuberculin antigens in the different formulations (calculated from IU/ml of IFN-γ secretions)

Sample No.	IFN- γ secretions (IU/ml)								% Response to tuberculin Ag $\frac{Ag - Nc}{Pc - Nc} \times 100$					
	CL	CD	S	M	T	H	Pc	Nc	CL	CD	S	M	T	H
1	8.698	-0.138	0.286	0.127	1.608	17.852	134.624	1.926	5.104	-1.555	-1.236	-1.356	-0.239	12.002
2	7.958	0.074	0.656	0.392	0.974	16.899	130.550	-0.085	6.156	0.122	0.567	0.365	0.810	13.001
3	1.820	1.608	2.190	2.296	1.820	12.508	96.899	-0.138	2.017	1.799	2.399	2.508	2.017	13.032
4	1.767	0.603	1.026	0.974	1.979	12.772	92.825	0.286	1.601	0.343	0.800	0.743	1.830	13.493
5	4.148	1.503	0.921	1.661	2.984	11.608	105.471	-0.455	4.346	1.848	1.299	1.998	3.247	11.389
6	4.942	0.815	0.709	0.180	2.243	10.339	111.026	-0.349	4.751	1.045	0.950	0.475	2.328	9.596
7	3.037	-0.349	3.302	1.503	2.032	9.280	103.619	0.286	2.663	-0.614	2.919	1.178	1.690	8.705
8	3.354	-0.032	2.561	0.762	1.344	4.360	109.651	-0.825	3.784	0.718	3.065	1.437	1.964	4.693
9	1.503	1.079	1.397	0.603	1.714	9.069	115.947	0.127	1.188	0.822	1.096	0.411	1.370	7.720
10	1.873	0.603	1.732	1.238	0.709	9.439	114.413	0.550	1.162	0.046	0.511	0.604	0.139	7.807
Average values	3.910	0.577	1.478	0.974	1.741	11.413	111.503	0.132	3.277	0.457	1.290	0.836	1.516	10.403

Pc: Positive control (Mitogen)
denoted a 100% response
Nc: Negative control (PBS)

Table 5 Summary of the assessment of tuberculin activities of the experimental formulations

Classified by type of analysis	Formula	Mean activity	Result
In vivo test (DTH test in guinea-pigs)	CL	7.80 mm.	
	S*	4.72 mm.	* Significant loss of activity
	M	7.02 mm.	
	T	6.88 mm.	
	H	7.87 mm.	
In vitro test (IFN- γ test in blood samples)	CL	3.28 %	
	S	1.29 %	
	M	0.84 %	
	T	1.52 %	
	H*	10.40 %	* Clearly shown for highest response

- Mean activity from DTH test is the average of reactions from 30 animals
- Mean activity from IFN-γ test is the average of % response to tuberculin from 10 samples, 100% denoted the response of mitogen in the test kit of IFN-γ assay

4. Stability study

The accelerated degradation of lyophilized formulations (M, T, H) and liquid formulation (CL) was performed to find out the residual tuberculin activities at each interval of storage: 0, 3, 6, 9 and 12 months at 4°c, 37°c and 56°c. The results of mean reactions obtained after injection of the samples into 30 guinea pigs were shown in Figure 12. Table 6 gave the results from bioassay in terms of % remaining activity of each formulation after storage in these conditions. The calculation for data in the table was done by using the activity (mean reaction in mm) of each formulation and the initial activity (mean reaction in mm) of liquid formulation prior to storage (at 0 month as 100 % activity). The data in Table 6 demonstrated the stabilities of 3 lyophilized formulations compared to the stability of liquid formulation. At time zero (no accelerated degradation), the activity of Haemaccel (H) resulted 100 % of the initial

activity of liquid formulation, whereas Mannitol (M) and Trehalose (T) retained about 90 %. Under the condition of accelerated degradation employed in 3 months, H retained highest activity about 97 % at 4°c, H and T retained highest activity of 100 % at 37°c and 56°c respectively. In 6 months, T retained highest activity about 90 % at every storage temperature. In 9 months, both T and H retained highest activity about 82 % at 4°c, H retained highest activity about 70% at 37°c and 56°c. In long term storage of 12 months, H retained highest activity about 70 % at 4°c and 37°c whereas T retained highest activity about 60 % at 56°c. The curves of % remaining activity (mm) and storage time (month) were plotted separately for each storage temperature from the data in Table 6 (as shown in Figure 13). It exhibited the profiles of remaining activity of each formulation after storage at 4°c, 37°c and 56°c for different length of time. From the result of 4°c, it was found that H was the most stable formulation in long term storage of 12 months.

Table 6 Percentages of tuberculin remaining activity of each formulation after storage in various conditions, calculated by using the initial activity of liquid formulation prior to storage (0 month) as 100% activity (Test in 30 guinea pigs per condition)

Time (month)	% Remaining activity											
	Liquid PPD (CL)			Mannitol (M)			Trehalose (T)			Haemaccel (H)		
	$4^{O}C$	$37^{O}C$	$56^{O}C$	$4^{O}C$	$37^{O}C$	$56^{O}C$	$4^{O}C$	$37^{O}C$	$56^{O}C$	$4^{O}C$	$37^{O}C$	$56^{O}C$
0	100.0	100.0	100.0	89.7	89.7	89.7	88.5	88.5	88.5	**100.0**	**100.0**	**100.0**
3	74.4	48.7	26.9	88.5	83.3	75.6	96.2	85.9	**100.0**	**97.4**	**100.0**	92.3
6	55.1	57.7	19.2	85.9	71.8	57.7	**91.0**	**87.2**	**87.1**	79.5	83.3	76.9
9	57.7	51.3	21.8	76.9	52.6	35.9	**82.1**	64.1	66.7	**82.1**	**73.1**	**71.8**
12	52.6	42.3	11.5	57.7	47.4	35.9	56.4	55.1	**56.4**	**69.2**	**67.9**	52.6

Highest activity in a group is in bold type

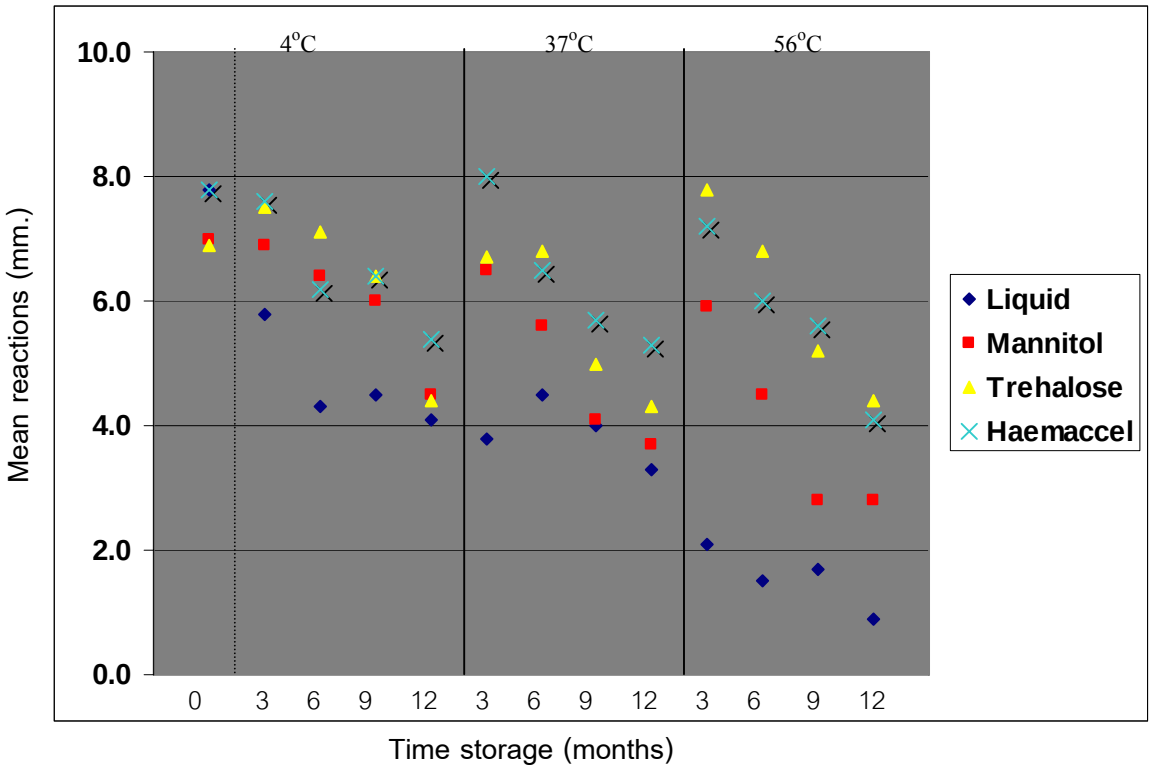

Figure 12 **Mean reactions of DTH responses obtained from injection of the samples into guinea pigs after storage in different conditions**

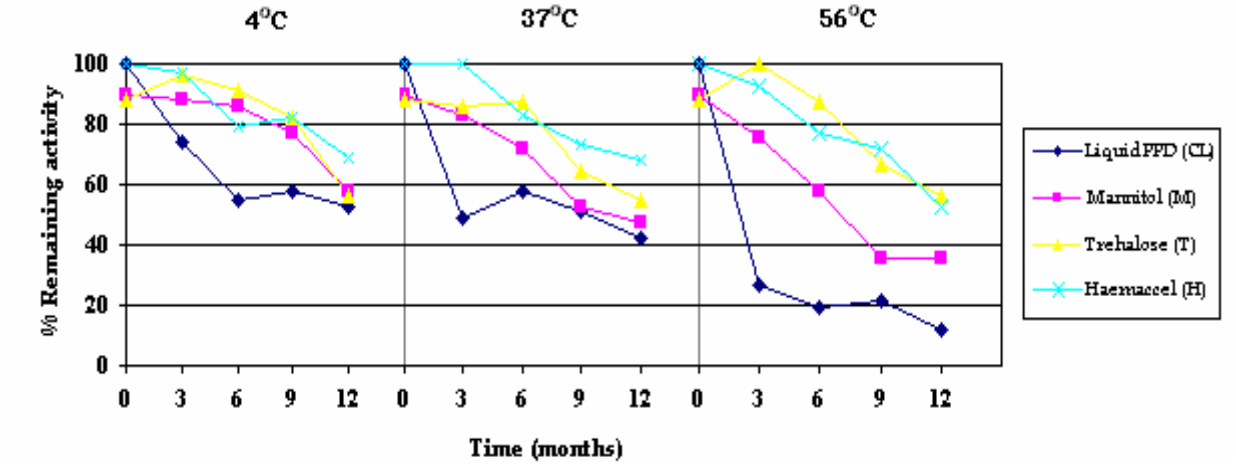

Figure 13 **Remaining activity of tuberculin PPD in each formula (as determined by bioassays) after storage at 4°C, 37°C, 56°C for different length of times**

CHAPTER V
DISCUSSION

The results in this experimental research have shown the efficacies of lyophilized tuberculin PPD which were produced from the designed formulations. They displayed the degree of DTH responses in biological assay and CMI responses by IFN-γ secretion assay. The biological activity and immunological activity of tuberculin PPD are due to the content of protein antigens which are present in the products after lyophilized formulating with various kinds of excipients.

Basing on the knowledge that there is no chemical method available that can be used for assessment of the potency or activity of tuberculin with satisfactory accuracy (22), the first assay was therefore performed by using biological method. In fact, tuberculin PPD is used as a diagnostic tool for *Mycobacterium tuberculosis* infection but PPD contains a number of antigens that can be shared with the vaccine strain (*Mycobacterium bovis* BCG), pathogenic strain and environmental mycobacteria (24, 25). In most laboratories, the biological assessment is performed in guinea pigs which can be sensitized with BCG or virulent tubercle bacilli. The potency value of tuberculin that obtained from guinea pig model is generally considered to be valid also for humans (22). The second is based on the assay for IFN-γ response as a method for detecting tuberculin activity. It is the quantitative method that has been developed and used as an *in vitro* test in comparison with DTH skin test (6). It was performed by using human blood of subjects who previously had DTH responses to tuberculin in Mantoux tests with the results of at least 5 mm diameter because this size of area of induration is the threshold for indication of a positive reaction (8).

The methodology of analysis for tuberculin activity in this research has been considered from the pathological point of view about the difference between DTH response and CMI response to mycobacterial infecion which has been noted that DTH is a process that destroys the unactivated macrophages within which tubercle bacilli are multiplying so that the bacilli are inhibited extracellularly but CMI is a process that activates macrophages to kill and digest the bacilli intracellularly (14). The analyses

therefore have to be confirmed by both responses in the animal and laboratory tests. In the other points of view about immune response to tuberculin, the antigen-specific T lymphocytes proliferate and release lymphokines, which mediate the accumulation of other cells at the site of injection (2). This response is DTH which is an accelerated immune inflammatory response that results in damage to host tissue, especially when local concentrations of tuberculin-like antigens are high (14). The tissue reaction seen in the tuberculin test is the result of the activity of lymphocyte mediators and the macrophages (10). It is also the result of the activity of tuberculin which is due to the amount of protein in that tuberculin preparation (21). In part of CMI response, IFN-γ is a marker of CMI reactivity and can be used for investigating the competence of antigen (28). Theoretically, lymphocytes from tuberculin positive blood already sensitized by mycobacterial antigens, when stimulated with tuberculin PPD, these memory T-cell lymphocytes secrete the cytokine IFN-γ that is measurable. This mechanism depends on the ability of tuberculin antigen in eliciting the responses. On the other hand, the ability of T-cells to respond to tuberculin reflects the cellular hypersensitivity. Antigen concentration is one of the factors that can influence the degree of responsiveness of a sensitive lymphocyte to the antigen (10). All of these describable points show the way to investigate the responses in both DTH and CMI in order to ensure the results of tuberculin activity shown in different methods of analysis.

The problem of performing CMI response assay is that it was not possible to determine the IFN-γ secretion level in guinea pig blood (from the same group of guinea pigs already tested with each of tuberculin formulation). If the guinea pig blood can be tested, it may point out the correlation of IFN-γ secretion with DTH reactions in the same kind of subject. This problem was caused by the fact that the reagent kit for Interferon-gamma test (QuantiFERON-TB) cannot be used to measure IFN-γ in guinea pig plasma as the antibodies used in the ELISA component of this reagent are species specific (only detect human IFN-γ). There is no cross reaction with guinea pig IFN-γ which is the awareness of the manufacturer. The IFN-γ standard used for reading off the amount of IFN-γ secretion in IU/ml is also human IFN-γ so that the investigation for IFN-γ responses had to be performed in human blood of subjects with

the criteria of positive reactions (diameter ≥5 mm) from Mantoux tests (8). The degree of IFN-γ response from the stimulation of tuberculin antigen in the product is interpreted as strength or activity of tuberculin in each individual formulation.

From the results of both analyses, it has been shown that the tuberculin activity as measured by DTH reaction and IFN-γ secretion from the formulation containing Haemaccel indicates the highest values. The *in vivo* test demonstrates peak of remaining activity of tuberculin PPD in Haemaccel formulation agreeing with the *in vitro* test which shows highest percentage of human IFN-γ in response to tuberculin PPD in Haemaccel formulation.

In the study of stability, the accelerated degradation test (ADT) was performed and evaluated on the result of excipient that acted as the most effective stabilizer among the excipients used in this group of study. It has been sought to determine the stability of tuberculin PPD by estimating the activities remained after various periods of storage at high temperatures that may give a good indication of how the products will behave at unfavourable temperatures encountered in storage and transport in the tropical areas. The use of biological assay for ADT was very complicated because it required a large number of animals. The stability of each lyophilized formulation was determined by comparison of the activity of lyophilized product after storage in short and long term periods at different temperatures with the initial activity of liquid product before lyophilization. From the observations of ADT, the results show that addition of Trehalose and Haemaccel to the tuberculin preparations can protect the final lyophilized products against activity loss in some conditions of storage. However, it is likely that Haemaccel formula retains more activity than Trehalose formula and thus constitutes a more durable lyophilized product in long term storage at 4°c for 12 months. Normally, 4°c is recommended for temperature storage of most of the biological products. Haemaccel is a gelatin polymer that can be used both as cryoprotectant and lyoprotectant and has the property of increasing protein solution viscosity and limiting protein structural movement (33). Haemaccel itself is a pharmacologically inert substance so that it did not interfere with the tuberculin reactions. In case of side effects, the skin test by injections of Haemaccel solution (EH formula) without PPD in guinea pigs did not show the appearance of urticarias or wheals after injections.

The results in this research give the information of the fantastically high activity of tuberculin PPD indicating the involvement of 0.3%Haemaccel addition in the formulation of lyophilized tuberculin PPD product.

CONCLUSION

The success of this study is the demonstration of the effectiveness of excipients in comparisons among selected excipients in this group of study. It has been found that Haemaccel is the most effective one which shows the best stability of tuberculin PPD and may lead to the longest shelf life. The Haemaccel formula should be appropriate for use in the development of lyophilized formulation. The most important thing is that whenever the new batch of lyophilized tuberculin PPD is prepared for use in human, the strength of tuberculin PPD must be confirmed that it is equivalent to 5 IU dose of PPD-S (46). To assure the strength of the new product of Haemaccel formula, further study should be carefully determined by the assay of potency of test tuberculin in guinea pigs (1). If it fails to meet the requirement for potency, the batch shall be rejected and then new trial batch of Haemaccel formula should be reproduced with the optimization of the volume of concentrate tuberculin PPD from the bulk of starting material in order to yield the acceptable tuberculin PPD activity for Mantoux test. It is very important that the newly produced tuberculin PPD should be finally standardized by bioassay in both tuberculin sensitized guinea pigs and humans. All commercially produced, stabilized tuberculin PPDs were required to be bioassayed and shown equivalent to 5 IU dose of PPD-S before marketing. The injection of 5 IU was recommended by Furcolow et al. in 1942, on the basis of the fact that it caused reactions in 99.6% of patients with active tuberculosis (17). In the manufacturer's practice, after finish the preparation of new product of tuberculin PPD, the potency dose bioequivalent to 5 IU of PPD-S is assayed for reactivity in the model of guinea pigs and the clinical testing is also conducted in humans in order to standardize the tuberculin product against the International reference standard. According to this concept, the lyophilized tuberculin PPD with the addition of 0.3%Haemaccel should be assayed along both ways.

In conclusion, this is the experimental research for development of the production of diluted tuberculin PPD required in lyophilized form. The results highlighted the most effective excipient for durable product, however it is the

comparison in only a group of excipients which have been sought from the knowledge about their properties involving the freeze drying of proteins. From this comparison study, Haemaccel represents candidate excipient in the formulations. It is highly probable for use 0.3% Haemaccel in the formulation of lyophilized tuberculin PPD in the future, but the production must be under consideration on optimizing the strength of solution to be bioequivalent to 5 IU of PPD-S. The standardization to the International reference is essential for meaningful interpretation of the Mantoux test result. Furthermore, the maintaining of 4°c cold chain for storage and transport is necessary even though it has been developed for the lyophilized product of long-life tuberculin PPD.

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APPENDIX

APPENDIX I

Basic data of skin reactions (mm) from 30 guinea pigs in biological assay

GP No.	CL	CD	S	M	T	H
1	8.0	2.5	0.0	5.0	5.0	4.5
2	3.5	2.0	0.0	5.5	6.5	7.0
3	5.0	3.5	0.0	8.5	9.0	7.0
4	7.5	0.0	9.0	8.0	8.5	10.0
5	9.5	0.0	6.5	10.5	10.5	11.0
6	9.5	5.5	10.5	8.5	10.0	10.0
7	9.5	8.5	8.0	11.0	11.0	11.5
8	8.0	5.0	11.0	9.0	8.0	9.0
9	9.0	10.5	0.0	11.0	10.5	12.0
10	5.0	9.0	0.0	4.5	4.5	6.5
11	6.0	9.5	6.5	9.0	6.5	10.0
12	7.0	5.0	3.5	4.0	4.0	5.0
13	8.0	9.0	0.0	5.0	6.0	8.5
14	7.0	8.5	6.0	5.0	4.5	5.0
15	9.5	8.5	0.0	7.0	7.5	8.5
16	10.0	5.0	6.5	0.0	0.0	6.0
17	3.5	7.5	4.5	6.0	3.5	3.5
18	6.5	5.5	8.0	9.5	9.0	0.0
19	9.5	6.5	0.0	9.5	8.0	11.0
20	8.0	4.5	6.0	6.0	5.0	8.5
21	6.5	8.5	2.0	6.5	5.0	6.5
22	8.5	7.5	7.5	7.0	7.0	11.0
23	9.0	8.5	10.0	6.5	4.5	6.5
24	10.5	2.5	7.0	11.0	10.5	10.0
25	8.5	5.5	4.0	9.0	9.5	9.5
26	7.0	9.5	4.0	6.5	7.0	7.5
27	8.5	10.5	8.0	7.0	7.5	8.0
28	9.0	3.5	8.0	5.5	6.5	8.5
29	8.5	8.5	5.0	9.0	6.5	6.0
30	8.5	7.0	0.0	0.0	5.0	7.0
Mean ± SD	**7.8 ± 1.81**	**6.3 ± 2.97**	**4.7 ± 3.71**	**7.0 ± 2.80**	**6.9 ± 2.54**	**7.8 ± 2.67**
Remaining	**-**	**81%**	**60%**	**90%**	**89%**	**100%**

APPENDIX II

Basic data from the bioassay of 4 formulas stored for 3 months at various temperatures
(Diameter of reaction in mm from injection of samples into BCG-vaccinated guinea pigs , n=30)

No. of Guinea pig	Liquid			Mannitol			Trehalose			Haemaccel		
	$4^{O}C$	$37^{O}C$	$56^{O}C$	$4^{O}C$	$37^{O}C$	$56^{O}C$	$4^{O}C$	$37^{O}C$	$56^{O}C$	$4^{O}C$	$37^{O}C$	$56^{O}C$
1	6.0	5.0	0.0	5.5	8.0	10.5	8.0	6.0	7.0	6.5	7.5	7.0
2	4.0	0.0	0.0	8.0	6.0	7.0	10.5	11.0	7.5	6.0	10.0	9.0
3	4.5	0.0	0.0	6.0	8.5	6.5	11.0	9.0	8.5	9.0	10.0	8.0
4	5.5	5.5	0.0	7.0	5.0	6.5	10.5	9.0	10.5	8.0	8.5	8.0
5	6.0	0.0	0.0	10.5	8.5	8.5	9.0	7.5	11.5	8.0	5.5	9.0
6	6.5	4.5	0.0	9.0	9.0	8.0	9.0	6.5	10.0	8.0	9.5	9.0
7	6.0	6.5	0.0	9.0	5.5	3.5	6.5	8.0	8.5	8.5	7.0	9.0
8	6.0	6.5	8.5	9.0	6.5	5.5	4.0	6.5	8.5	7.0	8.5	6.5
9	7.5	5.0	5.0	9.0	9.0	7.5	6.0	12.5	11.0	7.0	9.5	10.5
10	6.0	0.0	0.0	8.5	11.0	9.0	7.5	11.0	6.5	10.0	11.5	10.5
11	5.5	3.0	4.0	5.5	5.0	6.5	8.0	6.5	7.0	2.5	8.5	6.0
12	5.5	6.0	5.0	0.0	8.5	6.0	7.0	9.5	7.5	9.0	9.5	9.5
13	6.5	0.0	5.0	7.5	9.5	6.5	5.5	8.0	6.5	6.0	5.0	10.0
14	7.5	5.0	7.5	7.0	8.0	6.5	6.5	6.0	6.0	9.0	8.5	7.5
15	7.0	6.5	7.5	6.0	7.5	4.5	8.5	6.5	8.0	7.0	11.0	10.0
16	4.0	0.0	3.5	5.5	10.0	9.0	8.5	10.5	8.0	7.0	10.5	7.0
17	4.5	2.0	0.0	5.5	5.5	6.5	4.5	0.0	8.5	6.5	5.5	6.0
18	6.0	5.0	3.0	8.5	0.0	0.0	8.5	6.5	7.0	10.0	8.0	5.5
19	3.5	0.0	0.0	7.0	7.0	7.5	8.5	6.5	7.0	8.0	8.0	7.0
20	3.0	5.0	2.0	5.5	8.5	7.0	8.5	8.0	8.5	7.5	7.5	8.0
21	6.0	5.0	0.0	5.0	6.5	5.5	7.0	7.0	7.5	6.5	6.5	9.0
22	7.0	6.0	3.0	9.5	0.0	7.0	5.0	5.5	5.0	9.5	9.5	0.0
23	6.0	1.0	0.0	4.5	4.0	0.0	6.0	0.0	5.0	7.5	5.5	0.0
24	7.0	7.0	1.0	7.5	0.0	4.0	5.0	0.0	7.0	6.0	4.5	4.0
25	8.0	6.5	4.5	8.0	7.0	8.5	7.0	5.5	7.0	9.5	7.0	8.5
26	4.5	4.0	1.0	7.5	9.0	8.0	9.0	6.0	8.0	8.0	9.0	5.0
27	8.5	7.5	1.0	6.5	6.5	0.0	7.5	5.0	8.5	7.5	7.5	5.0
28	4.0	3.5	0.0	7.0	5.5	6.0	8.0	5.5	7.0	9.0	5.5	5.5
29	6.5	5.0	1.0	7.0	4.5	5.5	7.5	6.5	6.0	7.0	6.0	6.5
30	6.0	3.5	0.0	3.5	6.0	0.0	7.0	6.0	8.0	6.5	8.0	8.5
Mean	5.8	3.8	2.1	6.9	6.5	5.9	7.5	6.7	7.8	7.6	8.0	7.2
SD	1.34	2.55	2.65	2.09	2.80	2.78	1.74	2.96	1.55	1.54	1.87	2.61

APPENDIX III

Basic data from the bioassay of 4 formulas stored for 6 months at various temperatures
(Diameter of reaction in mm from injection of samples into BCG-vaccinated guinea pigs , n=30)

No. of Guinea pig	Liquid			Mannitol			Trehalose			Haemaccel		
	$4^{O}C$	$37^{O}C$	$56^{O}C$	$4^{O}C$	$37^{O}C$	$56^{O}C$	$4^{O}C$	$37^{O}C$	$56^{O}C$	$4^{O}C$	$37^{O}C$	$56^{O}C$
1	6.5	3.0	5.0	5.0	5.0	7.0	7.0	4.5	6.0	7.0	6.5	6.5
2	7.5	6.0	6.0	8.5	7.5	8.0	9.0	5.0	9.5	8.5	8.5	8.5
3	4.0	8.0	0.0	3.0	3.0	5.0	6.0	3.0	1.0	4.0	6.5	3.0
4	7.5	4.5	3.0	7.0	5.0	7.5	10.5	9.5	10.5	8.5	7.0	8.0
5	4.0	8.0	0.0	7.0	7.0	3.0	7.0	5.0	7.0	8.0	8.0	7.5
6	6.0	7.0	0.0	7.0	6.5	6.5	10.0	7.0	5.0	7.0	6.5	8.0
7	3.0	1.5	1.5	5.0	1.5	0.0	7.5	9.5	1.5	7.0	2.5	3.0
8	4.0	2.5	0.0	9.0	7.5	0.0	6.5	4.0	8.5	7.0	5.0	6.5
9	4.0	3.0	1.5	4.0	4.5	1.5	4.5	7.0	5.0	7.5	7.0	7.5
10	8.0	6.5	0.0	0.0	5.0	1.5	4.5	7.0	7.5	7.0	8.0	6.5
11	5.0	7.5	3.5	7.0	4.5	0.0	10.0	7.0	10.5	8.0	7.5	7.5
12	7.0	8.0	1.5	5.5	5.5	0.0	11.0	9.0	9.0	8.0	8.0	7.0
13	1.5	1.5	0.0	9.5	7.5	6.5	7.5	9.0	7.5	2.5	3.0	2.5
14	5.0	6.5	0.0	8.5	8.5	7.0	9.0	9.0	9.0	6.5	7.5	5.5
15	0.0	6.0	0.0	7.0	8.0	5.0	7.0	7.0	10.5	6.0	6.5	6.5
16	3.5	5.0	0.0	6.5	4.5	5.5	7.0	7.0	8.0	6.0	6.5	8.0
17	0.0	7.0	0.0	7.0	7.5	8.0	7.5	8.5	7.0	6.5	6.5	7.0
18	0.0	0.0	0.0	7.5	1.5	4.5	9.0	7.0	7.5	7.5	8.0	6.0
19	0.0	0.0	0.0	4.0	6.5	7.0	4.5	5.0	4.0	0.0	3.0	1.5
20	0.0	0.0	0.0	7.5	6.0	5.5	6.5	7.5	5.5	1.5	5.0	1.5
21	0.0	0.0	0.0	1.5	5.0	0.0	4.5	7.0	7.0	6.5	4.0	0.0
22	7.0	6.5	5.0	7.0	4.5	6.5	4.0	7.0	4.0	6.0	7.0	6.0
23	0.0	0.0	0.0	7.0	3.5	0.0	5.0	7.5	7.0	5.5	4.0	9.0
24	6.5	6.5	3.5	6.5	7.5	5.0	7.5	7.0	8.0	7.5	8.0	8.0
25	7.5	6.5	8.0	6.5	5.5	8.0	9.0	7.5	7.0	6.5	8.0	6.0
26	8.5	8.0	4.0	8.0	8.5	7.5	8.5	9.0	8.0	8.0	8.0	7.5
27	4.5	4.0	2.5	5.5	7.5	5.5	6.5	3.5	4.0	7.0	8.5	8.0
28	5.0	5.0	0.0	8.0	5.0	6.5	5.0	5.0	5.0	5.0	8.0	5.0
29	5.5	6.5	0.0	7.0	6.0	0.0	7.0	7.0	5.5	5.5	5.0	6.0
30	5.5	0.0	0.0	7.5	3.0	2.0	6.5	6.0	7.5	5.0	7.0	7.5
Mean	4.2	4.5	1.5	6.3	5.6	4.3	7.2	6.8	6.8	6.2	6.5	6.0
SD	2.84	2.92	2.24	2.12	1.91	3.00	1.94	1.77	2.40	2.00	1.75	2.33

APPENDIX IV

Basic data from the bioassay of 4 formulas stored for 9 months at various temperatures
(Diameter of reaction in mm from injection of samples into BCG-vaccinated guinea pigs , n=30)

No. of Guinea pig	Liquid			Mannitol			Trehalose			Haemaccel		
	$4^{O}C$	$37^{O}C$	$56^{O}C$	$4^{O}C$	$37^{O}C$	$56^{O}C$	$4^{O}C$	$37^{O}C$	$56^{O}C$	$4^{O}C$	$37^{O}C$	$56^{O}C$
1	6.0	6.5	3.5	5.0	5.0	5.0	7.0	6.0	4.0	7.0	5.5	4.0
2	5.0	5.0	5.0	9.0	5.0	2.5	8.5	5.5	2.5	9.0	2.5	3.0
3	2.5	0.0	0.0	8.0	0.0	0.0	7.5	4.0	0.0	5.0	6.0	6.0
4	6.5	5.0	6.0	8.5	5.0	4.0	5.5	6.5	0.0	6.0	6.5	0.0
5	4.5	0.0	0.0	5.5	4.5	0.0	7.0	0.0	0.0	5.0	5.0	5.0
6	6.0	3.5	3.5	6.0	5.0	2.5	6.0	6.0	5.5	5.5	0.0	6.0
7	0.0	0.0	0.0	4.0	0.0	0.0	5.5	5.5	0.0	5.0	6.5	5.5
8	4.0	3.0	4.0	4.5	5.0	0.0	6.5	2.5	5.0	5.0	6.0	6.5
9	5.5	6.0	5.0	5.5	0.0	0.0	9.0	0.0	4.0	7.5	0.0	4.0
10	2.0	0.0	0.0	2.5	6.0	0.0	7.5	0.0	5.5	5.0	5.0	3.0
11	1.0	0.0	5.0	0.0	3.5	6.5	6.0	0.0	5.0	5.0	4.5	4.5
12	6.5	10.0	4.5	10.0	10.0	7.0	10.0	9.0	10.5	8.0	10.0	9.5
13	5.5	5.0	0.0	5.5	4.5	0.0	5.0	4.0	5.0	7.5	6.0	5.5
14	5.0	8.0	2.0	8.5	2.0	4.0	5.5	6.0	6.5	6.5	6.0	10.0
15	5.0	3.0	0.0	7.5	0.0	5.0	9.0	8.0	8.0	8.0	8.5	4.5
16	3.0	4.0	5.0	5.0	0.0	1.0	6.5	3.0	6.0	7.5	5.0	7.0
17	6.5	7.0	0.0	5.5	4.5	1.0	8.0	6.0	7.5	8.0	6.5	7.5
18	6.0	5.0	0.0	6.0	1.0	2.0	3.0	4.0	3.0	4.0	3.0	1.0
19	4.0	0.0	0.0	6.0	3.0	2.0	4.0	4.0	7.0	6.5	5.0	6.0
20	6.0	6.5	0.0	9.0	8.5	8.0	8.5	8.5	8.5	9.0	8.5	8.0
21	6.5	4.0	0.0	6.5	6.5	6.5	4.5	5.5	7.5	8.5	5.0	6.5
22	7.5	6.0	3.0	6.0	5.0	0.0	7.5	6.5	6.5	8.0	7.5	7.5
23	6.5	6.5	3.0	9.5	8.0	7.0	9.5	9.0	9.5	7.5	8.5	8.5
24	4.5	6.5	0.0	5.5	3.5	2.0	7.0	6.5	7.5	5.0	6.0	7.5
25	6.0	6.5	0.0	8.5	6.5	6.0	8.0	6.5	6.5	7.5	8.0	8.0
26	3.0	0.0	0.0	4.0	0.0	3.0	4.0	3.0	4.0	4.0	3.0	2.5
27	2.0	3.0	0.0	5.0	8.5	6.5	6.0	5.0	6.0	5.5	4.0	3.0
28	5.0	6.5	0.0	5.0	7.0	0.0	6.0	7.0	9.0	5.5	9.0	8.0
29	3.0	3.0	0.0	4.5	0.0	0.0	0.0	7.0	7.0	5.0	7.5	4.0
30	2.5	0.0	0.0	4.5	4.0	3.0	3.5	4.5	0.0	4.0	7.0	5.5
Mean	4.6	4.0	1.7	6.0	4.1	2.8	6.4	5.0	5.2	6.4	5.7	5.6
SD	1.89	2.89	2.17	2.20	2.94	2.71	2.16	2.58	3.00	1.55	2.39	2.41

APPENDIX V

Basic data from the bioassay of 4 formulas stored for 12 months at various temperatures
(Diameter of reaction in mm from injection of samples into BCG-vaccinated guinea pigs , n=30)

No. of Guinea pig	Liquid			Mannitol			Trehalose			Haemaccel		
	$4^{O}C$	$37^{O}C$	$56^{O}C$	$4^{O}C$	$37^{O}C$	$56^{O}C$	$4^{O}C$	$37^{O}C$	$56^{O}C$	$4^{O}C$	$37^{O}C$	$56^{O}C$
1	3.0	2.0	0.0	0.0	0.0	0.0	3.0	4.0	0.0	0.0	2.0	0.0
2	0.0	0.0	0.0	3.5	0.0	0.0	4.0	3.0	0.0	0.0	0.0	0.0
3	6.0	5.0	3.0	3.5	0.0	3.5	3.0	8.0	3.5	5.0	0.0	2.0
4	2.0	0.0	0.0	3.0	0.0	0.0	2.0	3.0	4.0	3.0	4.0	5.0
5	5.5	4.0	0.0	7.5	3.0	8.5	7.5	8.5	7.0	8.5	7.5	7.5
6	0.0	3.0	0.0	2.0	0.0	2.0	3.0	0.0	2.0	3.0	0.0	3.0
7	3.0	5.0	2.0	3.0	0.0	0.0	5.0	7.0	2.0	3.0	5.0	0.0
8	6.0	5.0	0.0	0.0	3.0	4.0	3.0	2.0	0.0	5.0	5.0	3.5
9	5.5	6.0	3.0	0.0	0.0	0.0	5.0	0.0	0.0	6.0	5.0	5.0
10	0.0	0.0	0.0	0.0	0.0	0.0	4.0	7.0	0.0	0.0	0.0	0.0
11	6.0	1.0	5.0	6.5	6.5	0.0	5.5	5.0	7.0	8.0	7.5	6.0
12	4.5	4.0	0.0	5.0	4.5	5.5	3.5	6.0	4.0	4.0	5.0	3.5
13	4.0	3.5	0.0	5.0	4.5	2.0	0.0	0.0	4.0	3.5	3.0	0.0
14	6.5	5.0	0.0	7.5	6.5	6.0	5.5	6.0	0.0	8.0	5.5	8.5
15	1.0	4.5	0.0	1.0	2.0	0.0	1.0	0.0	4.0	6.5	6.5	1.0
16	6.0	3.5	0.0	5.0	7.5	5.0	9.5	1.0	4.5	3.0	6.5	6.5
17	4.0	0.0	0.0	6.0	3.0	5.5	5.5	4.5	6.0	6.5	6.5	3.0
18	6.0	1.0	0.0	4.0	7.0	0.0	2.0	5.5	5.0	6.5	6.0	5.0
19	6.0	4.5	0.0	1.0	1.0	1.0	7.5	7.0	6.0	6.5	6.5	7.5
20	6.5	6.0	5.0	8.5	8.0	6.0	9.0	8.0	8.5	8.5	9.0	6.5
21	3.0	2.0	0.0	4.5	6.5	0.0	1.0	0.0	7.0	7.0	6.0	5.0
22	5.0	0.0	0.0	6.0	4.0	1.0	4.0	1.0	5.0	4.5	4.0	6.0
23	6.0	1.0	0.0	6.5	3.5	5.5	5.5	6.0	4.0	8.0	6.5	7.5
24	1.0	5.0	0.0	6.5	5.0	3.5	6.5	5.0	7.0	5.5	8.0	1.0
25	6.5	7.0	4.0	9.5	8.5	6.5	5.5	8.5	8.5	8.0	7.5	8.5
26	7.0	7.0	2.0	8.0	8.5	6.0	9.0	10.0	11.5	9.5	9.5	6.5
27	5.0	5.0	0.0	5.0	8.5	5.0	4.5	5.0	7.0	7.0	7.5	6.5
28	2.5	0.0	0.0	6.5	4.0	0.0	0.0	0.0	8.5	7.5	6.5	0.0
29	5.0	6.5	2.0	7.0	6.5	6.0	6.5	7.0	5.0	7.5	7.5	8.0
30	1.0	3.0	0.0	2.0	0.0	0.0	1.0	0.0	1.0	3.0	6.0	1.0
Mean	4.1	3.3	0.9	4.5	3.7	2.8	4.4	4.3	4.4	5.4	5.3	4.1
SD	2.25	2.34	1.59	2.78	3.12	2.77	2.61	3.19	3.12	2.66	2.66	2.99

APPENDIX VI

The percentage of activity remaining
(as estimated from reactions of the initial activity of liquid)

Products were stored at $4^{O}C$ for 0 , 3 , 6 , 9 and 12 months

Time (months)	Liquid (CL)		Mannitol (M)		Trehalose (T)		Haemaccel (H)	
	Activity (mm)	% Remaining	Activity (mm)	% Remaining	Activity (mm)	% Remaining	Activity (mm)	% Remaining
0	7.8	100	7.0	89.7	6.9	88.5	7.8	**100**
3	5.8	74.3	6.9	88.5	7.5	96.2	7.6	**97.4**
6	4.3	55.1	6.4	82.1	7.1	**91.1**	6.2	79.5
9	4.5	57.7	6.0	76.9	6.4	**82.1**	6.4	**82.1**
12	4.1	52.6	4.5	57.7	4.4	56.4	5.4	**69.2**

Products were stored at $37^{O}C$ for 0 , 3 , 6 , 9 and 12 months

Time (months)	Liquid (CL)		Mannitol (M)		Trehalose (T)		Haemaccel (H)	
	Activity (mm)	% Remaining	Activity (mm)	% Remaining	Activity (mm)	% Remaining	Activity (mm)	% Remaining
0	7.8	100	7.0	89.7	6.9	88.5	7.8	**100**
3	3.8	48.7	6.5	83.3	6.7	85.9	8.0	**100**
6	4.5	57.7	5.6	71.8	6.8	**87.2**	6.5	83.3
9	4.0	51.3	4.1	52.6	5.0	64.1	5.7	**73.1**
12	3.3	42.3	3.7	47.4	4.3	55.1	5.3	**67.9**

Products were stored at $56^{O}C$ for 0 , 3 , 6 , 9 and 12 months

Time (months)	Liquid (CL)		Mannitol (M)		Trehalose (T)		Haemaccel (H)	
	Activity (mm)	% Remaining	Activity (mm)	% Remaining	Activity (mm)	% Remaining	Activity (mm)	% Remaining
0	7.8	100	7.0	89.7	6.9	88.5	7.8	**100**
3	2.1	26.9	5.9	84	7.8	**100**	7.2	92.3
6	1.5	19.2	4.5	75.6	6.8	**87.2**	6.0	76.9
9	1.7	21.7	2.8	35.9	5.2	66.7	5.6	**71.8**
12	0.9	11.5	2.8	35.9	4.4	**56.4**	4.1	52.6

BIOGRAPHY

NAME	Mrs. Thipchuta Bharnthong
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PLACE OF BIRTH	Bangkok, Thailand
INSTITUTIONS ATTENDED	Mahidol University, 1968-1972: Bachelor of Science (Medical Technology) Mahidol University, 1999-2004: Master of Science (Medical Technology)
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