A LYOPHILIZED FORMULATION TO EXTEND THE SHELF-LIFE OF TUBERCULIN PPD

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Abstract. This study was aimed to develop a dry purified protein devirative (PPD) preparation to extend the shelf-life of tuberculin PPD. Five percent sucrose (S), 6.5% mannitol (M), 2.5% trehalose (T) or 0.3% Hemaccel (H) was added to each formulation. *In vivo* and *in vitro* analyses were carried out to determine the efficacy of the lyophilized products. In the *in vivo* test, the delayed type hypersensitivity (DTH) responses of the lyophilized preparations were compared to the liquid preparation (CL) after injection into BCG vaccinated guinea pigs. The preparations of H, M, T, and S generated DTH responses of 100, 90, 89, and 60%, as compared to the response of CL, respectively. There was no loss of tuberculin activity in the H formula. A statistically significant difference in activity was found between S and CL ($p<0.05$). The cellular test for IFN-γ secretions was performed using the whole blood of human subjects screened for DTH response to tuberculin PPD Mantoux tests. The detection of IFN-γ secretions was done using ELISA and the efficacy was expressed in terms of percentage of IFN-γ responses to the tuberculin antigens. The results of CL, H, M, T and S were 3.28, 10.40, 0.84, 1.52 and 1.29%, compared to mitogen stimulation, respectively. The lyophilized H, M and T formulations and the liquid CL were studied for their shelf-life stabiliy. Accelerated degradation was done by storing the samples at higher temperatures of 37ºC and 56ºC for 3, 6, 9 and 12 months. All the tuberculin PPD solutions were injected into BCG vaccinated guinea pigs at the end of each storage period and the activity of each solution was evaluated. The formulation with the Hemaccel as excipient gave a superior response than the others at the normal storage temperature of 4ºC for 12 months. Therefore, Hemaccel provides protection for PPD activity. This supports the potential for the development of lyophilized tuberculin PPD with the addition of 0.3% Hemaccel to extend shelf life.

INTRODUCTION

Tuberculin is commonly used as a tool for diagnosis and epidemiological surveys of tuberculosis. The purified protein derivative (PPD) of tuberculin is a reagent for the Mantoux skin test to determine the DTH response to tuberculin in an infected or immune subject. Tuberculin is a liquid preparation of killed organisms of *Mycobacterium tuberculosis*; protein was shown by Siebert in 1941 to be the active agent of tuberculin. This led the way to the preparation of PPD (Siebert and Glen, 1941). It is known that PPD is active in eliciting a hypersensitivity reaction in the subject sensitized with mycobacteria. An infected individual probably retains sensitive to tuberculin as long as viable bacilli and memory T cells remain within the body. The size and intensity of the reaction depends on the amount of tuberculin injected and the degree of sensitivity in the subject (National Tuberculosis Advisory Council, 1979). The potency of tuberculin is assayed by estimating the protein content in the solution by intradermal injection of PPD into BCG vaccinated guinea pigs. The DTH results in a localized induration at the injection site. Its diameter is measured at 24 hours after injection into the guinea pig (WHO Expert Committee on Biological Standardization, 1987). The standard Mantoux test in humans involves injecting 0.1 ml containing 5 IU of PPD solution intradermally on the volar surface of the forearm, and measuring the size of the induration at 48 to 72 hours. The strength of 1 IU is defined as the biological activity of tuberculin contained in 0.000028 mg of PPD-S, which has been adopted by the World

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Health Organization as the International Reference Standard for Mammalian Tuberculin PPD (Affronti *et al*, 1969; Huebner *et al*, 1993). Tuberculin testing is performed using a small amount of freshly diluted tuberculin. The Mantoux test has been largely used by hospitals and tuberculosis control programs. Ready for use diluted tuberculin is necessary due to the increased screening for tuberculosis infections. Centralized preparation to permit standardization and reduce the risk of contamination is needed (Magnus *et al*, 1958). This practice has to focus on maintaining a stable, active form of tuberculin. It has been reported that diluted tuberculin PPD has no significant loss of activity when stored at 2º-4ºC for 6 months (Wong and Quyang, 1940; Schmidt-Rohr and Winnewisser, 1956; Edward *et al*, 1963). This duration includes the time required for quality control tests after the preparation and shipment of the product, consequently the actual shelf-life of diluted PPD in the hands of users is about 4 to 5 months, which is inadequate when compared to the shelf-life of other pharmaceutical products. Highly diluted tuberculin PPD results in a shorter shelf-life and remains a serious problem for producers and users. The question whether ready for use diluted tuberculin PPD can withstand prolonged storage is important. In Thailand, diluted tuberculin PPD for use in DTH skin testing (Mantoux method) is produced at the QSMI laboratory of the Thai Red Cross, namely the Thai Red Cross Tuberculin PPD (PPD-TRC) and is distributed to users throughout the country. The concentrated solution of tuberculin PPD is diluted to 100 units per ml or 10 units per dose. The strength of the 10 unit dose of PPD-TRC is equivalent to a 5 IU dose of PPD-S (Bharnthong *et al*, 2002). The diluted PPD solution is prepared monthly because it becomes unstable after dilution. In this study, extending the shelf-life of diluted tuberculin PPD was determined using lyophilization, adding excipients as stabilizers.

MATERIALS AND METHODS

Tuberculin purified protein derivative (PPD) and excipients

A concentrated tuberculin PPD (24,180 mg of total protein content (~487,500 units) per container; Lot No. 57/58) purchased from Chiron S.p. A (Seina), Italy was used as a starting material for the preparation of PPD stock solution. Briefly, the protein powder was precisely reconstituted with 5 ml diluent (phosphate buffered solution containing 5 ppm Tween 80 and 0.3% phenol, pH 7.38) to achieve 97,500 units/ml final concentration.

Four kinds of stabilizing agents, including sucrose (Merck, Germany), mannitol (Merck), trehalose (Merck) and Hemaccel (3.5% polygeline, Hoechst Marion Roursel Deutschland, Germany) were selected and used as excipients (Heller *et al*, 1999).

Experimental animal for *in vivo* testing

Guinea pigs (White, Albino), weighing 400-450 g, which have been sensitized intradermally on the abdomen with 0.05 mg BCG vaccine (Lot No. 653, QSMI, Thai Red Cross, Thailand), were used as subjects to test for tuberculin activity. The assay was performed 6 weeks after vaccination.

Reagent kit and blood samples for *in vitro* testing

An assay of Tuberculin Interferon-gamma (IFN-γ) secretion was performed using a whole blood Interferon-gamma kit (QuantiFERON-TB, Cellestis, Victoria, Australia). Heparinized venous blood samples were collected from 5 human subjects who had responded to tuberculin PPD antigen with a diameter of reaction ≥5 mm verified on the Mantoux test.

Lyophilization of tuberculin PPD

Process design for lyophilization. To achieve satisfactory characteristics for the lyophilized product, the following parameters were used: 1) loading and pre-freezing: loading temperature +20ºC (after shelf cooling for 1.5 hours); decrease to 0ºC in 20 hours; freezing at -35ºC; cooling rate of 0.6ºC/minute; and a holding time of 4.5 hours; 2) primary drying: heating rate of 0.03ºC/minute; pressure $<5.0 \times 10^{-2}$ mbar; and a holding time of 4 hours; 3) secondary drying: increase to +20ºC over 5 hours; heating rate of 0.06ºC/minute; pressure $<5.0 \times 10^{-2}$ mbar; and a holding time of 13 hours; 4) total cycle time: 48 hours.

Preparation of designed formulations. A stock solution of tuberculin PPD (97,500 units/ml) was

diluted to 100 units/ml and used as the liquid formulation (CL). A double strength solution (200 units/ml) was prepared for the lyophilized formulations. Addition of sucrose (S), mannitol (M), trehalose (T) and Hemaccel (H) to the PPD solutions was performed to yield final concentrations of 5.0, 6.5, 2.5 and 0.3%, which were then referred to as formulas S, M, T and H, respectively. Next, aliquots of 0.5 ml from each formulation were separately inserted into 2 ml vials. These vials were then half closed with rubber stoppers and put into the lyophilizer, model FD20 (Heto Lab Equipment, Denmark), for 48 hours. Once the complete cycle was reached, the rubber stoppers were closed immediately under high vacuum in the lyophilizer chamber and sealed tightly with aluminum caps. Prior to use, the sample was reconstituted using 1.0 ml of water for injection (WFI) to yield a concentration of 100 units/ml, the same as the liquid sample. For comparison, the lyophilized PPD without excipients (CD) and lyophilized excipients without PPD (ES, EM, ET and EH) were prepared as controls.

Moisture and pH determination. The residual moisture content of the lyophilized products was determined immediately after lyophilization using the karl-Fisher titration (Orion model AF8, Orion, USA). The pH of the tuberculin PPD solutions after reconstitution with WFI were measured using a pH meter (Orion model 520A, Orion, USA).

Biological assay for tuberculin activity

Assay of tuberculin activity was carried out by detection of delayed-type hypersensitivity (DTH) responses upon intradermally injection of PPD in sensitized guinea pigs. Briefly, 0.1 ml of each formulation of PPD solution (CL, CD, S, M, T and H) was injected ramdomly on the abdomen skin of the guinea pigs (n = 30). Injection of the lyophilized excipients without PPD (ES, EM, ET and EH) was performed for comparison. The DTH response was analyzed after 24 hours. Longitudinal and tranverse measurements of the diameters (in mm) of indurations at the injection sites were performed. Statistical analysis of the reaction sizes comparing the lyophilized formulas (CD, S, M, T and H) and the liquid formula (CL) was done using the paired *t*-test. It is noteworthy that the acceptable formula for stability study denoted no significant difference ($p > 0.05$).

Tuberculin interferon-gamma (IFN-γ) assay

Detection of IFN-γ secretion was performed using the QuantiFERON-TB kit. In this study, the solutions (5 μg/ml) of CL, S, M, T and H were used as test antigens instead of using the PPD antigen supplied by the manufacturer. One millimeter of whole blood was mixed with 100 μl (0.5 μg) of each tuberculin solution and subsequently transferred to the microplate. The plate was then shaken for 1 minute and incubated for 20 hours at 37ºC. After incubation, the supernatant was collected, and 50 μl was evaluated to quantify the IFN-γ level (IU/ml) by using ELISA, and compared to the standard human recombinant IFN-γ concentration (NIH Ref: Gx901-902-535). The tuberculin activity for each of the products (CL, CD, S, M, T and H) was expressed as a percentage of the response to the tuberculin PPD antigen calculated from the following equation:

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

Where Ag, Pc and Nc are the Tuberculin antigen, Positive control (in the presence of mitogen) and Negative control (PBS), respectively.

Stability study

To test the stability of the lyophilized formulas, the accelerated degradation assay was performed by monitoring the tuberculin activity upon exposure to various temperatures. The samples were subjected to elevated temperatures of 37ºC and 56ºC for 3, 6, 9 and 12 months, prior to determining the remaining activity by the DTH skin test. The remaining activity of the samples stored at 4ºC were also investigated for comparison.

RESULTS

The design of the lyophilized formulations of tuberculin PPD were performed using Sucrose (S), Mannitol (M), Trehalose (T) and Hemaccel (H) as candidate excipients and the concentrations in the solutions prior to lyophilization varied as described in the methods. After lyophilization, all the products for the S, M, T and H formulations showed satisfactory physical characteristics. The residual moisture content of each

Table 1
Comparison of the efficacies of lyophilized formulations and the liquid formulation of diluted PPD by DTH responses in guinea pigs. (Each mean is the average for reactions from 30 tests).

Formulations	Mean size of reactions in mm	Remaining activities (%)
CL	7.8	-
CD	6.3	83
S	4.7	60
M	7.0	90
T	6.9	89
H	7.8	100
ES, EM, ET, EH	no reaction	0

CL = liquid formulation, S = sucrose, M = mannitol, T = trehalose, H = Hemaccel, CD = formulation without excipients, ES = sucrose without tuberculin, EM = mannitol without tuberculin, ET = trehalose without tuberculin, and EH = Hemaccel without tuberculin.

Table 2
Statistical analysis of tuberculin activities by paired *t*-test as expressed by mean size of reaction of the lyophilized formulation compared to the liquid formulation.

		Lyophilized formulations				
	CL	CD	S	M	T	H
Mean reaction in mm	7.8	6.25	4.72	7.02	6.88	7.83
Standard deviation	1.81	2.97	3.71	2.8	2.54	2.67
p (T ≤ t) two-tail		0.02	0.00006	0.17	0.07	0.94

CL = liquid formulation, CD = lyophilized PPD without excipients, S = sucrose, M = mannitol, T = trehalose, and H = Hemaccel.

lyophilized product (S=3.6%, M=1.2%, T=6.7% and H=8.5%) were within the accepted limit of 10% (Wang, 2000). The pH in formulation was between 6.5-7.5, which is the normal range for a tuberculin solution (WHO Expert Committee on Biological Standardization, 1987). Analysis was performed using *in vivo* and *in vitro* tests as follows.

Biological activity of tuberculin

The results of tuberculin activity in the different lyophilized formulations (S, M, T, H), the liquid formulation (CL), and in the control (CD) revealed different levels of DTH responses in the immune animals. The reactions in 30 guinea pigs were measured at 24 hours after injection with the tuberculin PPD solutions. The mean size of the reaction in millimeters and the percentage of remaining activities are shown in Table 1. The activities of lyophilized formulations were calculated by comparison with the initial activity of the liquid formulation. They varied according to the efficacies of the excipients. The H showed the highest activity (100% remaining activity) among those experimental excipients. The results presented in Table 2 show that there are significant differences between CD and CL, and between S and CL ($p < 0.05$). No significant differences were present when the M, T, and H formulas were compared with CL. The negative controls for the ES, EM, ET and EH formulas, which lacked tuberculin PPD, showed no reactions at the injection sites.

Induction of interferon-gamma

The tuberculin activities of the lyophilized formulations were analysed by measurement of IFN-γ levels. Based on the standard curve of linear correlation ($r = 0.999$), the IFN-γ secretions for each tuberculin PPD antigen contained in these lyophilized formulations can be seen in Table 3. For the comparison of IFN-γ levels, the

Table 3
IFN-γ secretions and percentage of IFN-γ responses to lyophilized PPD formulations.

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	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

CL = liquid formulation, CD = lyophilized PPD without excipients, S = sucrose, M = mannitol, T = trehalose, H = Hemaccel, Pc = mitogen control, Nc = negative control, and Ag = tuberculin antigen.

percentage of responses to the tuberculin antigens were calculated from the values. The resullts presented in Table 3 show that the H formula was superior in its ability to induce IFN-γ secretion (10.4%).

Extended stability of lyophilized formula

The accelerated degradation tests on the lyophilized formulations (M, T, H) and the liquid formulation (CL) were performed by storage at 4º, 37º and 56ºC for 3, 6, 9 and 12 months. The activity of each formulation after storage was compared to that at zero time, which is shown in Fig 1. The calculation was done using the initial activity of the liquid formulation prior to lyophilization as 100% activity. The data demonstrate the 3 lyophilized formulations remained stable when compared to the initial activity of the liquid formulation. At time zero, the activity of H resulted in 100% of the initial activity, whereas M and T retained about 90% activity. When stored for 3 months at 4ºC, H retained 97% activity, the highest. H and T retained the highest activities of 100% at 37ºC, and 100% at 56ºC, respectively. At 6 months, T retained the

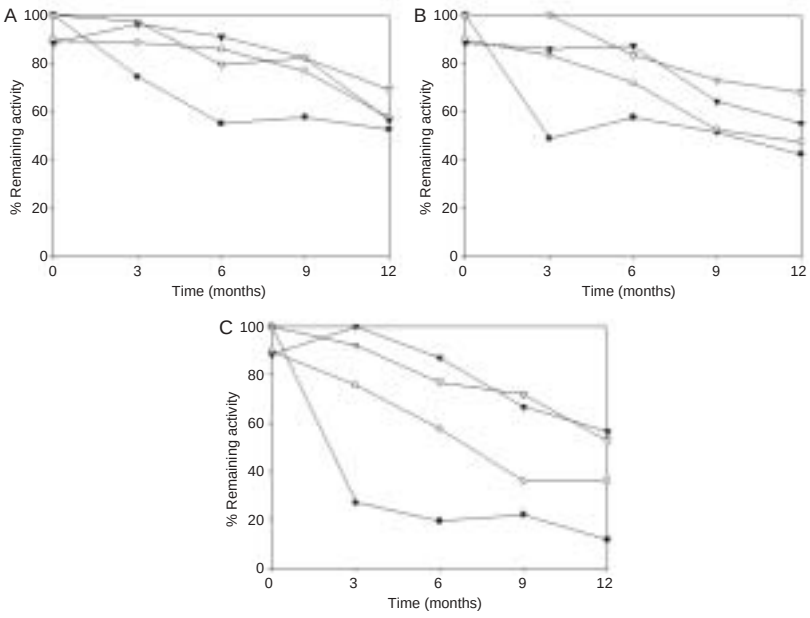

Fig 1–Remaining tuberculin activity versus time at 4ºC (A), 37ºC (B) and 56ºC (C) of diluted PPD formulas. ● = Liquid PPD, ○ = Mannitol, ▼ = Trehalose, and ▽ = Hemaccel.

highest activity, of about 90%, at each storage temperature. At 9 months, both T and H retained the highest activity, about 82% at 4ºC, while H retained the highest activity, of about 70%, both at 37ºC and 56ºC. At 12 months, H retained the highest activity, of about 70%, at 4ºC and 37ºC, whereas T retained the highest activity, of about 60%, at 56ºC.

DISCUSSION

We have presented here a design formula to extend the shelf life of diluted tuberculin PPD. The lyophilized formula of tuberculin PPD with 0.3% Hemaccel generates a 100% recovery of activity after lyophilization. The Hemaccel formula also retains its activity for up to 12 months at 4ºC. Trehalose also had no significant difference from Hemaccel when stored long term. However, 10% decline in activity was noted immediately after lyophilization with trehalose. Hemaccel is a pharmacologically inert gelatin polymer, which has been applied as a cryoprotectant and lyoprotectant to increase protein viscosity and limit structural degradation (Wang, 2000). Trehalose is a disaccharide, and is quite expensive. In addition, 0.3% Hemaccel lyophilized PPD promoted the best stimulation of IFN-γ secretion from T lymphocytes. Therefore, Hemaccel is preferable. The quality of diluted tuberculin PPD is a significant factor in the diagnosis of patients with tuberculosis (Lee and Holzman, 2002). The injection of 5 IU has been recommended. In practice, the potency dose bioequivalent to 5 IU of PPD-S is needed to be conducted with each production batch. Guinea pigs and clinical testing in humans for DTH can be used to standardize tuberculin PPD products (Hansen *et al*, 1964; Oettinger *et al*, 1997).

In conclusion, lyophilized tuberculin PPD with 0.3% Hemaccel extends shelf-life and is a ready to use tuberculin PPD that can retain its activity for at least 12 months. Maintaining a 4ºC cold chain for storage and transportation is necessary.

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REFERENCES

Affronti LF, Caprio JJ, Edwards PQ, *et al*. What is PPD-S? A statement by the Committee on Diagnostic Skin Testing. *Am Rev Respir Dis* 1969; 99: 460-1.

Bharnthong T, Kongwatana C, Premchaiporn P, *et al*. A comparison of PPD-TRC and PPD-S by Mantoux test. *Thai J Tuberc Chest Dis* 2002; 23: 23-31.

Edwards LB, Cross FW, Hopwood L. Effect of duration of storage on the potency of dilutions of PPD antigens. *Tubercle* 1963; 44: 153-61.

Hansen OG, Lindqvist K, Waaler H. Assessment of the potency of tuberculin in humans and guinea-pigs. *Bull World Health Organ* 1964; 31: 171-82.

Heller MC, Carpenter JF, Randolph TW. Protein formulation and lyophilization cycle design: Prevention of damage due to freeze-concentration induced phase separation. *Biotechnol Bioeng* 1999; 63: 166-74.

Huebner RE, Schein MF, Bass JB Jr. The tuberculin skin test. *Clin Infect Dis* 1993; 17: 968-75.

Lee E, Holzman RS. Evolution and current use of the tuberculin test. *Clin Infect Dis* 2002; 34: 365-70.

Magnus K, Guld J, Waaler H, Magnusson M. Stability of purified tuberculin in high dilution. *Bull World Health Organ* 1958; 19: 765-82.

National Tuberculosis Advisory Council. Review and recommendations: The tuberculin test. Canberra: Australian Government Publishing Service, 1979.

Oettinger T, Holm A, Haslov K. Characterization of the delayed type hypersensitivity- inducing epitope of MPT64 from *Mycobacterium tuberculosis. Scand J Immunol* 1997; 45: 499-503.

Schmidt-Rohr H, Winnewisser T. Stability of stored tuberculin dilutions under various conditions. *Munch Med Wochenschr* 1956; 98: 190-3.

Seibert FB, Glen JT. Tuberculin purified protein derivative; preparation and analyses of a large quantity for standard. *Am Rev Tuberc* 1941; 44: 9-24.

Wang W. Lyophilization and development of solid protein pharmaceuticals. *Int J Pharm* 2000; 203: 1-60.

WHO Expert Committee on Biological Standardization. Requirements for tuberculins. Thirty-sixth report. *WHO Tech Rep Ser* 1987; 745.

Wong SC, Quyang G. Studies on stability of diluted purified tuberculin. *Proc Soc Exp Biol* 1940; 45, 83.