CHAPTER III
EXPERIMENTAL

Materials

All chemicals and reagents were analytical or pharmaceutical grades and were used as received.

1. Aluminium hydroxide gel (Bureau of Veterinary Biologic, Department of Livestock Development, Thailand)
2. Ammonium acetate (Lot F3B273, Ajax Chemicals, Australia)
3. Anti-Beijing mouse IgG (JE) (Lot pool A1+A3, , Government Pharmaceutical Organization, Thailand)
4. Bicinchoninic acid disodium AR (BCA, Lot 053K532V, Sigma, USA)
5. Bicinchoninic acid kit (Sigma, USA)
6. *Bordetella pertussis* antibody (Lot 9L36401, Biodesign International, USA)
7. Citric acid AR (Lot 0086978, Fisher Scientific, UK)
8. Diphtheria Toxoid (Lot DV46002, Government Pharmaceutical Organization, Thailand)
9. Diphtheria antibody (Lot 1H21303, Biodesign International, USA)
10. di-Sodium hydrogen phosphate AR (Lot FZG096, Ajax Chemicals, Australia)
11. Dithizone AR (Lot 434553/1, Fluka, Switzerland)
12. Etylenedinitrilo (EDTA) AR (Lot 7727 KMTP, Mallinckrodt Chemical, USA)
13. Gelatin (medium gel strength) (Lot 424331/1, Fluka, Switzerland)
14. Glacial acetic acid (Merck, Germany)
15. Horseradish peroxidase-goat anti-mouse IgG (H+L) conjugate (Lot 40286774, Zymed Laboratories, USA)
16. Horseradish peroxidase-labeled anti-Beijing mouse IgG (Lot 181202, Government Pharmaceutical Organization, Thailand)
17. Hydrochloric acid solution 37%, AR grade (Labscan Asia Co., Ltd., Thailand)
18. Hydrogen peroxide 30% AR (Lot 749938D8, Panreac Quimica SA, Spain)

19. Japanese Encephalitis virus antigen (Lot JVJ48013-1 , Government Pharmaceutical Organization, Thailand)
20. Japanese Encephalitis virus reference antigen (Lot 197, Government Pharmaceutical Organization, Thailand)
21. O-Phenylenediamine (Lot 40788994, Zymed Laboratories, USA)
22. Potassium chloride AR (Lot F1G253, Ajax Chemicals, Australia)
23. Potassium dihydrogen phosphate AR (Lot F1F125, Ajax Chemicals, Australia)
24. Rabbit anti-horse IgG (whole molecule) peroxidase conjugate (Lot 034K4780, Sigma, USA)
25. Sodium carbonate AR (Lot 479307, Carlo Erba, Italy)
26. Sodium chloride AR (Lot F2C273, Ajax Chemicals, Australia)
27. Sodium ethylmercurithiosalicylate (thimerosol) AR (Lot 436657/1, Fluka, Switzerland)
28. Sodium hydrogen carbonate AR (Lot AF 310196, Ajax Chemicals, Australia)
29. Sodium hydroxide pellets AR (Lot B131198 214, Merck, Germany)
30. Sodium tartrate dihydrate dibasic AR (Lot 3176, Riedel-deHaën, Germany)
31. Sulfuric acid (Merck, Germany)
32. Tetanus antitoxin (Lot TC44002, Government Pharmaceutical Organization, Thailand)
33. Tetanus Toxoid (Lot 063, Government Pharmaceutical Organization, Thailand)
34. Tween 20 AR (Lot 448707/1, Fluka, Switzerland)
35. Whole cell *Bordetella pertussis* antigen (Lot PV45006, Government Pharmaceutical Organization, Thailand)
36. Zinc sulfate AR (Lot F2K005, Ajax Chemicals, Australia)

Instruments

1. Analytical balance (Mettler Toledo AG204, USA)
2. Autoclave (Hirayama MFG. Corp., Tokyo, Japan)
3. Centrifuge (centrifuge 5810, Eppendorf, Germany)
4. Fourier transform Infrared spectrometer (Spectrum one, Perkin Elmer, USA)
5. Laser diffractrometer (Mastersizer S, Malvern Instruments, UK)
6. Magnetic stirrer (Variomax multipoint, Komet, Taiwan)
7. Micropipet (Socorex, Switzerland)
8. Microplate reader (VICTOR^3, Perkin Elmer, USA)
9. Multichannel pipet (Socorex, Switzerland)
10. pH meter (model 210, Thermo Orion, USA)
11. X-ray diffractrometer (D8 Discover, Bruker AXS, Germany)
12. Scanning electron microscope (JSM-5410LV, JEOL, Japan)
13. Shaking Incubator (Lab Tech, USA)
14. Shaking water bath (PolyScience, USA)
15. UV-VIS spectrophotometer (Jasco V-530, Schimidzu, Japan)
16. Vortex mixer (model G-560E, Scientific Industries, USA)

Methods

Aluminium hydroxide gel (AH) had greater adsorptive capacity than aluminium phosphate gel (Gupta, 1998; Shi et al., 2001; Baylor et al., 2002 and Sripongsarn, 2005). Therefore, AH was chosen as the adjuvant in this study. Prior to the preparation of the adjuvant stock solution, the content of aluminium had to be determined.

1. Aluminium content assay

AH was assayed for aluminium content by back titration method as aluminum hydroxide gel (USP 25). Briefly, the accurately measured quantity of gel, equivalent to about 1.5 g of AH and hydrochloric acid were gently heated until solution was completed. After cooling, the solution was diluted with water to 500 ml. The solution of 20 ml was added with 25 ml of edetate disodium titrant and 20 ml of acetic acid-ammonium acetate buffer TS. After heating to near the boiling point for 5 minutes, the solution was cooled and added with 50 ml of alcohol, 2 ml of dithizone TS, and finally, titrated with 0.05 M zinc sulfate VS until the color changed from green-violet to rose-pink.

2. Preparation of aluminium containing adjuvant stock solutions

AH stock solution was prepared to have aluminium content of 1.67%w/v in 0.85%w/v normal saline solution (NSS) at pH 6.5, which was the optimal pH for antigens adsorption (Sripongsarn, 2005). AH gel was accurately weighed equivalent to about 1.67%w/v in beaker and diluted with 0.85%w/v NSS. The final pH was adjusted to required pH by the addition of hydrochloric acid or sodium hydroxide. The solution was transferred to volumetric flask and adjusted volume to 25 ml with 0.85%w/v NSS.

3. Adsorptive capacity of single antigen on adjuvant

In this experiment, adsorption of *Bordetella pertussis* on aluminium containing adjuvant was not performed and studied because there was no difference between unadsorbed and adjuvanted pertussis vaccine with regard to protection against disease. The adjuvant effect of AP or AH on the mouse intracerebral potency of whole cell pertussis vaccine is controversial (Gupta et al., 1993; Gupta et al., 1998).

3.1 Adsorption procedure

AH at fixed concentration of 1.67%w/v and at pH 6.5 was transferred to microtube. The required amount of 0.85%w/v NSS and antigen (e.g. diphtheria toxoid (DT), tetanus toxoid (TT) or JE antigen (JE)) (as listed in Table 10-12) were eventually added in the microtube and swirled with vortex immediately. Then the mixture was swirled for 30 minutes at 37°c, 120 rpm in a shaking incubator. After that the mixture was centrifuged at 3,000 rpm (Sripongsarn, 2005) in order to separate the aluminium particles so the supernatant was collected to assay the protein content by BCA or micro BCA method (Seeber et al., 1991; Masood et al., 1994; Shakhshir et al., 1994; Shakhshir et al., 1995; Rinella et al., 1996; Heimlich et al., 1999; Shi et al., 2002; Morefield et al., 2005). The standard procedure was followed. The standard and enhanced protocols were used depending on how much protein remained in solution.

The adsorptive capacity was calculated from the linearized Langmuir isotherm for the adsorption of each antigen.

$$\text{Adsorptive capacity} = \frac{\text{mg of maximum protein adsorbed}}{\text{mg of Al}} \quad \ldots\ldots\ldots\ldots [1]$$

Table 10 The composition of DT adsorbed preparation

1.67%w/v AH (µl)	DT (µl)	0.85%w/v NSS (µl)
50	300	650
50	400	550
50	500	450
50	600	350
50	700	250
50	750	200
50	800	150
50	850	100
50	900	50
50	950	-

Table 11 The composition of TT adsorbed preparation

1.67%w/v AH (µl)	TT (µl)	0.85%w/v NSS (µl)
20	100	880
20	200	780
20	300	680
20	400	580
20	500	480
20	600	380
20	700	280
20	800	180
20	900	80
20	950	30

Table 12 The composition of JE adsorbed preparation

1.67%w/v AH (µl)	JE (µl)	0.85%w/v NSS (µl)
20	50	930
20	100	880
20	200	780
20	300	680
20	400	580
20	500	480
20	600	380
20	700	280
20	800	180
20	900	80

3.2 Protein analysis

BCA and micro BCA assay

The bicinchoninic acid (BCA) and micro BCA assay is a colorimetric assay for total protein. It is based on chemical principle similar to those of the biuret and Lowry assays but the Micro BCA assay is extremely sensitive and optimized for use with dilute protein samples. For BCA, Standard Working Reagent (S-WR) is prepared by mixing 100 volume of Reagent A, consisting of an aqueous solution of 1% BCA-Na_2, 2% $Na_2CO_3.H_2O$, 0.16% Na_2 tartrate, 0.4% NaOH and 0.95% $NaHCO_3$, with 2 volume of Reagent B, consisting of 4% $CuSO_4.5H_2O$ in deionized water. For micro BCA, Micro-Reagent A (MA) consists of an aqueous solution of 8% $Na_2CO_3.H_2O$, 1.6% Na_2 tartrate, 1.6% NaOH and sufficient $NaHCO_3$ to adjust the pH to 11.25. Micro-Reagent B (MB) consists of 4% BCA-Na_2 in deionized water. Micro-Reagent C (MC) consists of 4 volumes of 4% $CuSO_4.5H_2O$ plus 100 volume of Micro-reagent

B. Micro-Working Reagent (M-WR) consists of equal volume of MC and MA. The color of S-WR and M-WR is apple green (Smith, 1985).

A standard solution containing an accurate amount of bovine serum albumin (BSA) 1 mg/ml was diluted to 8 dilutions with final concentration of 100, 200, 300, 400, 500, 600, 700 and 800 µg / ml for BCA and 3, 5, 10, 15, 20, 25, 30 and 35 µg/ml for micro BCA. The standard assay procedure of BCA and micro BCA consisted of mixing 1 volume of standard sample with 20 volume of S-WR and equal volume of standard sample with M-WR, respectively. Color development for BCA proceeded within 2 hours at room temperature, while micro BCA was 1 hour at 60° C. The absorbance was measured at the wavelength of 562 nm versus a reagent blank for BCA and versus deionized water for micro BCA. For micro BCA, the reagent blank was measured the same as the sample.

The concentration versus average absorbance from triplicate assays was plotted. The relationship between absorbance and concentration was calculated. The concentration of unknown could then be determined from the plot of concentration and absorbance obtained for standard protein.

The result of adsorptive capacity was the quantity of 1.67%w/v AH for adsorption of each antigen in combined formulation.

4. Adsorption of single antigen on adjuvant at various processing variables

For each antigen, DT, TT, JE, various processing variables were investigated.

(A). Effect of mixing speed

The mixtures were mixed at 200, 300, 400 and 500 rpm by magnetic stirrer.

(B). Effect of temperature

The processing temperatures were 5 ±1, 15±1, 25 ±1 °C in a circulated chamber by chiller and 37 ± 1 °C by water bath.

(C). Effect of mixing time

The adsorption was performed for 1, 5, 12 and 24 hour.

4.1 Adsorption procedure

Each antigen (equivalent to adsorbed DT 30 Lf, adsorbed TT 6 Lf and adsorbed JE 0.35 antigen unit /0.5 ml /dose) and 1.67%w/v AH from the adsorptive capacity (from section 3) were transferred to the circulated chamber and adjusted volume to 3 ml with 0.85%w/v NSS. The adsorption by mixing was performed at investigated various processing variables. The percentage of adsorption (% adsorption), from assaying protein content by the same method aforementioned (from section 3.2) was calculated.

$$\% \text{ adsorption} = \frac{(\text{total protein content} - \text{unadsorbed protein}) \times 100}{\text{total protein content}} \quad \ldots\ldots\ldots\ldots [2]$$

The effect of the optimal processing variables (e.g. mixing speed, temperature and mixing time) on adsorption which had the optimal %adsorption value were chosen for further study.

5. Adsorption of combined antigens on adjuvant

The compositions of combined formulation were diphtheria toxoid (DT), tetanus toxoid (TT), *Bordetella pertussis* (PT) and JE antigen which were equivalent to adsorbed DT 30 Lf, adsorbed TT 6 Lf, PT 20 O.U. (2×10^{10} cell), adsorbed JE 0.35 antigen unit. The formulation also consisted of a preservative, 0.01%w/v thimerosal and 1.67%w/v AH (to be equivalent to sum of usage for each adsorbed antigen) not

more than 0.85 mg aluminium / 0.5 ml (from United States Food and Drug Administration guidelines) (Baylor et al., 2002).

5.1 Effect of combination process

Adsorption processes of antigens on 1.67%w/v AH, using optimal processing variables (result from section 4) were performed by two procedures.

5.1.1 Separate adsorption

Each antigen (DT, TT and JE) was individually mixed with its adjuvant by magnetically stirring at the optimal processing variables and then all was transferred to another mixing container. PT and thimerosal were eventually added and the volume was adjusted with 0.85%w/v NSS. The combined preparation was mixed with the same optimal processing variables for 30 minutes. The combined preparation was collected to analyze the amount of each antigen by enzyme linked immunosorbent assay (ELISA).

5.1.2 Competitive adsorption

The optimal processing variables were chosen similarly to the separate adsorption. DT, TT, PT, JE antigens and 1.67 %w/v AH were transferred and magnetically stirred together in a mixing container. Finally, thimerosal was added and the volume was adjusted with 0.85%w/v NSS, then mixed for another 30 minutes. The preparation was collected to analyze the amount of each antigen by ELISA.

5.2 Enzyme linked immunosorbent assay (ELISA)

DT, TT and PT were examined for their content by competitive ELISA (Hozbort et al., 1995) whereas JE antigen was examined by direct sandwich ELISA. (Morita, 1989; WI of viral vaccine division, GPO)

5.2.1 Indirect antigen competition

The amount of DT, TT and PT were analyzed as followed, the 96 well ELISA microtiter plate was coated with 100 µl per well of DT optimal concentration in coating buffer overnight at 4 ± 1 °C to allow the coating antigen adsorbed to the well. The plate was thoroughly washed three times with phosphate buffer saline with tween 20 (PBST) pH 7.4 and allowed to dry. The 100 µl of blocking solution, 3% gelatin in PBST, was added to each well of the coated plate and incubated for 1 hour at room temperature.

The standard antigen and test samples, which were five fold steps serial dilutions, were incubated (pretitrated) simultaneous outside the plate with DT antibody which directed against antigen on the plate. DT antibody was diluted to optimal concentration with diluent buffer. The plate was thoroughly washed three times with PBST and allowed to dry. The 100 µl of each pretitrated serial dilution of samples and standards were added into triplicate wells of the coated plate. Diluent buffer was incubated with DT antibody and was used for control, whereas diluent buffer was used for blank by adding 100 µl per well. The plate was incubated at room temperature for 1 hour. The plate was washed with PBST for three times and allowed to dry. The 100 µl of a horseradish peroxidase-goat anti-mouse IgG (H+L) conjugate, diluted to optimal concentration with diluent, was added to each well and incubated for 1 hour at room temperature. The plate was washed again with PBST for three times. The 100 µl of the substrate, O-phenylene diamine (OPD), at a concentration of 1 mg per 12 ml of a citrate buffer plus 12 µl of 30% hydrogen peroxide, was added to each well. The plate was covered in the dark and incubated for 30 minutes at room temperature for color development. The 50 µl of 4 N sulfuric acid was added to each well to stop the reaction. The plate was gently shaken and the optical density (OD) was read at wavelength 490 nm using a microplate reader. The procedure was shown in Figure 6. The amounts of antigen content were calculated from standard curve which was plotted between percentage of antigen binding and ln protein nitrogen concentration.

The ELISA procedures of TT and PT were the same as of DT. Standard curve of DT, TT and PT were conducted as five fold serial dilutions.

$$\% \text{ antigen binding} = [1 - (b/b_0)] \times 100 \quad \ldots\ldots\ldots\ldots [3]$$

b = sample optical density

b_0 = control optical density

The optimal condition of DT was coating antigen concentration 10 µg/well, dilution of primary antibody (1°Ab) 1:500 and secondary antibody (2°Ab) 1:10,000. Moreover, the optimal condition of TT was coating antigen concentration 10 µg/well, dilution of primary antibody (1°Ab) 1:10,000 and secondary antibody (2°Ab) 1:10,000. The suspension of PT antigen could be immediately coated on the plate without sonicated. The optimal condition of PT was coating antigen concentration as 10^{11} cell/well, dilution of 1°Ab as 1: 4 and dilution of 2°Ab as 1: 5,000 (Sripongsarn, 2005). The testing process for determination of the optimal dilution of coating antigen, primary antibody (1°Ab) and secondary antibody (2°Ab) was performed in this study by using these aforementioned values. The standard curve was plotted between percentage of antigen binding and ln concentration or the optimal optical density which was used for the ELISA analysis of diphtheria toxoid, tetanus toxoid and *Bordetella pertussis*.

5.2.2 Direct sandwich

The amount of JE antigen was analyzed as followed, the 96 well ELISA microtiter plate was coated with 100 µl per well of anti-Beijing mouse IgG dilution 1 : 6,500 in carbonate - bicarbonate coating buffer pH 9.6 overnight at 4 ± 1°C to allow the coating antibody adsorbed to the well. The plate was thoroughly washed three times with phosphate buffer saline with tween 20 (PBST) pH 7.4 and allowed to dry. The 100 µl of blocking solution was added to each well of the coated plate and incubated for 1 hour at room temperature. The plate was thoroughly washed three times with PBST and allowed to dry. The test samples and reference vaccines were two fold steps serially diluted with diluent and 100 µl of each sample was added

into triplicate wells of the coated plate. Diluent buffer was used for blank by adding 100 µl per well. The sample plate was incubated at room temperature for 1 hour. The plate was washed with PBST for three times and allowed to dry. The 100 µl of a horseradish peroxidase-labeled anti-Beijing mouse IgG, dilute 1: 800 with diluent, was added to each well and incubated for 1 hour at room temperature. The plate was washed again with PBST for three times.

After that, the procedures were the same process as in section 5.2.1 except the stop reaction process which used 50 µl of 4 N sulfuric acid. The procedure was shown in Figure 5. The amounts of antigen content were calculated from standard curve which was plotted between ln optical density and ln dilution.

5.2.3 Cross reaction analysis

This analysis was conducted in order to examine the cross over reaction between the one antibody and other components in preparation (antigens, aluminium adjuvant, buffer and thimerosol).

All components in preparation were examined with individual antibody. It showed that diphtheria primary antibody had no cross over reaction with TT, PT, JE, AH, buffer and thimerosal. TT, PT and JE had the same results as DT. It indicated that the other components in preparation had no effect to interfere the results of samples in ELISA analysis. In other words, if the sample had no reaction with primary antibody, the optical density of sample would be close to the optical density of control which was used as reference (Sripongsarn, 2005).

6. Stability study

The combined preparations of DTP-JE which were formulated as separate adsorption (section 5.1.1) and competitive adsorption (section 5.1.2) were stored at temperature 2-8 °C for 4 months in order to evaluate the content of each antigen by randomly sampling every 1 month interval for analysis by ELISA method (section

5.2.1 for DT, TT and PT; section 5.2.2 for JE antigen). Moreover, the physical appearances of the preparations were observed initial and 4 month stored preparation.

7. Evaluation of materials and preparations

7.1 Scanning electron microscopy

The combined preparations (which were prepared by separate and competitive adsorption procedure at optimal processing variables) and 1.67%w/v AH were centrifuged until the supernatant was cleared. The supernatant was taken off and the precipitates were coated with gold using ion sputtering prior to the microscopic examination. The surface morphologies of combined preparations were observed by scanning electron microscope (SEM) and compared with the surface morphology of plain AH.

7.2 Fourier Transformed Infrared spectrometry

The combined preparations (which were prepared by separate and competitive adsorption procedure at optimal processing variables) and 1.67%w/v AH were centrifuged until the supernatant was cleared. The supernatant was taken off and the precipitates were sampled to assay. The infrared spectrum of adjuvant was used to identify and characterize this material. Infrared spectra of combined preparations were measured by pasting the samples on the potassium bromide cell and performed in the range of 4000 - 450 cm^{-1} and compared with pure AH adjuvant.

7.3 X-ray diffractrometry

The X-ray diffraction band of 1.67%w/v AH was used to identify and characterize this material. X-ray diffraction patterns of the combined preparations by separate and competitive adsorption procedure at optimal processing variables were investigated by using an X-ray diffractrometer and compared with the X-ray diffraction patterns of plain AH adjuvant.

The samples were centrifuged until the supernatant was cleared. The supernatant was taken off and the precipitates were sampled to assay. The precipitates were firmly packed in the cavity of a thin rectangular quarts slide by the other slide. The glass slide was taken off and the prepared sample was exposed to the X-ray beam in the X-ray diffraction chamber. The samples were irradiated with monochromatized Cu Kβ radiation at the speed of 0.04° per minute from 5° - 60° in the term of 2θ angles. The voltage, and current used were 30 kV, and 30 mA, respectively.

7.4 Particle size distribution analysis

The particle size of combined preparations which were adsorbed on the adjuvant at optimal condition, were prepared by separate and competitive adsorption. The combined preparations were performed by laser diffractrometry (LD) with pump speed 1500, ultrasonic displacement 15 and compared with particle sizes of 1.67% w/v AH.

The ultrapure water was used as the medium for the measurement of the particle size. The samples were triplicate measured and showed as average particle size. The results of LD reported the percentile sizes for 10%, 50% and 90% which expressed as d (0.1), d (0.5) and d (0.9) respectively and the width of the distribution, which was the measure of the absolute deviations from the median expressed as uniformity.

8. Statistical analysis

The means %adsorption values of each antigen on 1.67%w/v AH by using the various processing variables and the antigen content of stability were studied by analysis of variance (ANOVA). The means particle size distribution of two combined procedure preparations and 1.67%w/v AH were statistically evaluated by paired student's *t* - test. Results were considered statistically significant if $p<0.05$.