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Quadrivalent Combined Vaccine, Including Diphtheria Toxoid, Tetanus Toxoid, Detoxified Whole Cell Pertussis, and Hepatitis B Surface Antigen

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Abstract Various factors, such as the adsorption pH, adjuvant dose, and adjuvant age, which affect the adsorption degree and immunogenicity of an antigen, were investigated. In addition, the effect of pH, antigen content, and adjuvant content on immunogenicity was also studied through animal experiments. Within the ranges studied, a low pH for adsorption, freshly preformed gel, and low pH formulation for the combined DTwP-HepB vaccine were preferrable for the adsorption of the antigens. In addition, a higher DT content was found to have a positive effect on the HBsAg immunogenicity in the combined vaccine. Accordingly, considering the factors affecting the adsorption rate and immunogenicity of the antigens, a novel DTwP-HepB vaccine (40 Lf/ml of diphtheria toxoid, 15 Lf/ml of tetanus toxoid, 20 OU/ml of detoxified whole cell pertussis, 24 μg/ml of HBsAg, 24 μg Al/ml of $Al(OH)_3$ gel, 776 μg Al/ml of $AlPO_4$ gel, and pH 7.1) was developed, whose immunogenicity was comparable to the case of administrating, separately and simultaneously, a combined DTwP vaccine (40 Lf/ml of diphtheria toxoid, 15 Lf/ml of tetanus toxoid, 20 OU/ml of detoxified whole cell pertussis, 300 μg Al/ml of $AlPO_4$ gel, and pH 7.1) and mono HepB vaccine [Hepavax®, 24 μg/ml of HBsAg and 500 μg Al/ml of $Al(OH)_3$ gel], which satisfies the potency criteria of the K-FDA for a combined DTwP vaccine and mono HepB vaccine.

Key words: Immunogenicity, separately simultaneous administration, combined vaccine

Diphtheria, tetanus, pertussis, and hepatitis B are all globally distributed diseases and have caused much human suffering for a long time. To efficiently and conveniently protect people from these diseases, a combined DTwP vaccine has been marketed all over the world since the 1950s [10], along with mono HepB vaccine prepared from the plasma of hepatitis B virus-infected people or from safer recombinant yeasts [9]. However, the separate administration of a combined DTwP vaccine and mono HepB vaccine is inconvenient and has various disadvantages. Therefore, a combined DTwP-HepB vaccine is needed to decrease the number of immunizations, reduce the delivery cost, increase the vaccine coverage, and specifically immunize populations who are inaccessible due to geographic factors and/or individual tendencies. However, despite this important need, studies on the formulation of a combined DTwP-HepB vaccine have been scarce. The biggest problem in developing an efficient combined DTwP-HepB vaccine is a low HBsAg immunogenicity caused by a poor incompatibility between the HBsAg and DTwP components [1, 7]. Hence, the development of a combined DTwP-HepB vaccine with a higher HBsAg immunogenicity is urgently required.

Accordingly, the current study was undertaken to develop a new formulation of a combined DTwP-HepB vaccine, including diphtheria toxoid, tetanus toxoid, detoxified whole cell pertussis, and hepatitis B surface antigen (HBsAg). Various important factors, such as the adsorption pH, adjuvant age, adjuvant content, and formulation pH, which can affect the adsorption degree and immunogenicity of an antigen, were investigated. Furthermore, various formulations with different pHs, antigen contents, and adjuvant contents were compared in terms of the geometric mean anti-HBsAg titer and potencies of all the antigens. Finally, a new formulation for a combined DTwP-HepB vaccine was established, which not only showed a comparable HBsAg immunogenicity to the separate and simultaneous administration of a combined DTwP vaccine and mono HepB vaccine in animal experiments, but also satisfied the potency criteria of the K-FDA for all the antigens.

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MATERIALS AND METHODS

Preparation of Toxoid Bulks, Detoxified Whole Cell Pertussis Bulk, HBsAg Bulk, and Combined DTwP-HepB Vaccines

The diphtheria toxin was excreted into a culture medium of the *Corynebacterium diphtheriae* Park Willium strain and subsequently detoxified by formalin. This diphtheria toxoid was then purified by the combination of ultrafiltration, diafiltration, $(NH_4)_2SO_4$ precipitation, and gel permeation chromatography. The tetanus toxin was excreted from the *Clostridium tetani* Harvard strain, then formalin-detoxified, and purified using a similar method to that used to purify the diphtheria toxoid. The whole cell pertussis was prepared by culturing *Bordetella pertussis*. The culture broth was then centrifuged, and the resulting cell pellet suspended in saline and inactivated at 56°C for 30 min to obtain the detoxified whole cell pertussis bulk. The HBsAg secreted into the periplasmic space of recombinant *Hansenula polymorpha* was purified by sequential processes of cell disruption, ionic chromatography, ultrafiltration, diafiltration, ultracentrifugation, and gel permeation chromatography.

The combined DTwP-HepB vaccines were then formulated as follows; The diphtheria and tetanus toxoid bulks were separately adsorbed on a freshly preformed-$AlPO_4$ gel to make the diphtheria toxoid-adsorbed bulk and tetanus toxoid-adsorbed bulk, respectively, while the HBsAg bulk was adsorbed on an $Al(OH)_3$ gel (Alhydrogel®, Superfos Biosector, Denmark) to make the HBsAg-adsorbed bulk. For the antigen adsorption, a mixture of the antigen bulk and the alum gel was gently stirred at 4°C for 3 h, then incubated at 4°C overnight. The diphtheria toxoid-adsorbed bulk was first mixed with the tetanus toxoid-adsorbed bulk and HBsAg-adsorbed bulk, then with the detoxified whole cell pertussis bulk, and, to create the desired alum gel concentration, the $AlPO_4$ gel was finally added. The final pH of the combined DTwP-HepB vaccine formulation was adjusted by the addition of NaOH or HCl.

Preparation and Characteristics of Aluminum Phosphate Gel

To prepare the $AlPO_4$ gel in-house, 0.45 M of Na_3PO_4 in distilled water was fed into 0.8 M $AlCl_3$ in distilled water until the pH of the mixture reached around 4.5, then the final pH was adjusted to 5.5 with 5 N NaOH. The concentration of the resulting $AlPO_4$ gel was about 3,000 μg Al/ml. The morphological and physicochemical characteristics of the freshly preformed-$AlPO_4$ gel were then investigated. The PZC (point of zero charge) determined using a Zetameter (3Plus, Zetameter Inc., U.S.A.) was pH 6.7, which was within the pI range of a normal $AlPO_4$ gel [3], while the morphology (picture not shown) taken by transmission electron microscopy had a structure with a network of platy particles identical with a commercial $AlPO_4$ gel (Adju-phos®, Superfos Biosector, Denmark) [8]

Degree of Adsorption

The degree of adsorption of the antigens in the antigen-adsorbed bulks was assayed using a 30 cm long size exclusion silica-based column (G3000SW, TSK-GEL, TosoHaas, U.S.A.) embedded in an HPLC (Jasco, Japan). The buffer and flow rate used were 40 mM PBS and 0.5 ml/min, respectively. One hundred Lf/ml of the diphtheria toxoid, 15 Lf/ml of the tetanus toxoid, and 24 μg/ml of HBsAg were used as the external standards for quantifying the degree of adsorption. Small volumes of the antigen-adsorbed bulks were centrifuged at 7,000 rpm for 2 min, then 100 μl of the supernatants and 100 μl of the external standards were separately injected into the column and their respective areas compared.

The degree of adsorption of each antigen in the combined DTwP-HepB vaccine was calculated from the desorption degree of each antigen measured by a Western blot analysis. For the desorption degree of the diphtheria toxoid, tetanus toxoid, and HBsAg, an equine anti-diphtheria IgG and rabbit anti-horse IgG conjugated to alkaline phosphatase (AP) [Sigma, U.S.A.], equine anti-tetanus IgG and rabbit anti-horse IgG conjugated to AP (Sigma, U.S.A.), and goat anti-HBsAg IgG and rabbit anti-goat IgG conjugated to AP (Sigma, U.S.A.) were used as the primary and secondary antibodies, respectively. The supernatants from the combined DTwP-HepB vaccines were subjected to SDS-PAGE (10–20% Tricine gel, Novex, San Diego, U.S.A.), and all the proteins were completely transferred onto a nitrocellulose membrane. Whenever the vaccine samples were loaded on the SDS-PAGE gel, the four standard samples prepared by serial dilution were also loaded at the same time. The resulting immunoreactive bands for the standards were then used as the calibration curve for quantifying the antigens desorbed from the vaccine samples. The immunoreactive bands were scanned using a ScanJet IICX/T (Hewlett Packard, U.S.A.) and subsequently analyzed using analytical imaging software, Gel-Pro Analyzer 2.0 (Media Cybernetics, Silver Spring, U.S.A.).

Immunogenicity Assay

The HBsAg immunogenicity and protective anti-HBsAg antibody rate (percentage of mice above 10 mIU/ml of protective anti-HBsAg IgG level) for the combined DTwP-HepB vaccines were measured using 30 SPF-grade ICR mice weighing 20–22 g. Each mouse was subcutaneously immunized with 0.5 ml of the combined DTwP-HepB vaccine and bled after 4 weeks. The anti-HBsAg IgG titer in each serum was assayed using the EIA method (Ausab IMx system, Abbott Laboratories, U.S.A.). From the assay results, the geometric mean (GM) anti-HBsAg

IgG titer and protective anti-HBsAg antibody rate were then calculated.

To determine the immunogenicity of the diphtheria toxoid, tetanus toxoid, and whole cell pertussis, the potency was measured based on the following animal experiments (*minimum requirements for biological products in Korea, K-FDA*). For the diphtheria potency (or tetanus potency), five guinea pigs weighing 300-400 g were separately immunized with 0.75 ml of the combined DTwP-HepB vaccine, bled by heart puncture after 35 days, and the sera pooled. Two-fold and four-fold diluted antisera and a reference diphtheria antitoxin (1 IU/ml, NIBSC; reference tetanus antitoxin for tetanus potency, 1 IU/ml, NIBSC) were mixed with an equal volume of a reference diphtheria toxin (1 l+/ml, GCVC, Korea; reference tetanus toxin for tetanus potency, 1 l+/ml, GCVC, Korea). Next, two guinea pigs weighing 270-330 g (6 mice weighing 18±2 g were used for the tetanus potency) were each subcutaneously injected with 2 ml (0.2 ml for the tetanus potency) of one neutralized solution, and then, the neutralized antibody titer (diphtheria or tetanus potency) was calculated based on the time of death of the animals.

The whole cell pertussis potency of the combined DTwP-HepB vaccine was measured using SPF-grade ICR mice. The combined DTwP-HepB vaccine and a reference whole cell pertussis vaccine (11.9 IU/ml, GCVC., Korea) were diluted 8-, 40-, and 200-fold. Each dilution group was composed of 20 mice. All the mice were challenged into the brain with 0.025 ml of live *B. pertussis* 18323 (about 200 LD_{50}) 3 weeks after being intraperitoneally immunized with 0.5 ml of the diluted vaccine. Fourteen days after the challenge, the whole cell pertussis potency was statistically calculated based on the number of surviving mice.

The HBsAg potency was determined by measuring the ED_{50} value of the combined DTwP-HepB vaccine and a reference HepB vaccine (Hepavax®, GCVC, Korea). The combined DTwP-HepB vaccine and reference HepB vaccine were diluted 16-, 64-, 256-, and 1024-fold. Each dilution group, composed of 20 SPF-grade ICR mice, was intraperitoneally immunized with 1 ml of the diluted vaccine, then bled after 28 days. The number of seroconversed sera in each group was assayed by the EIA method (Anti-HBs 3.0 kit, Monolisa, U.S.A.). Based on the results, the ED_{50} values for the combined DTwP-HepB vaccine and reference HepB vaccine were measured, thereby calculating the relative HBsAg potency of the combined DTwP-HepB vaccine.

In the case of administrating a combined DTwP vaccine and mono HepB vaccine separately and simultaneously, the potency of the diphtheria toxoid, tetanus toxoid, whole cell pertussis, and HBsAg was measured using the same method as that used for the combined DTwP-HepB vaccine, except that the two vaccines were separately and simultaneously injected into different sites in the experimental animals.

Results and Discussion

Isoelectric pH of Diphtheria Toxoid, Tetanus Toxoid, and Aluminum Phosphate Gel, plus Optimal Adsorption pH of Antigens

It is already known that pH is a crucial factor in the adsorption of antigens on adjuvants like alum gels [4]. Therefore, various experiments were conducted to determine the optimal pH for adsorbing the tetanus and diphtheria toxoids on the freshly preformed $AlPO_4$ gel. Prior to the adsorption experiments, to select the experimental pH range, the isoelectric points of the adjuvant and antigens were measured as follows. The PZC of the freshly preformed $AlPO_4$ gel and pI values of the diphtheria and tetanus toxoids were assayed using a Zetameter, measuring the surface charge of the colloid particles, and an IEF gel (Novex, Invitrogen, U.S.A.), respectively. The result indicated that the PZC was 6.7 for the freshly preformed $AlPO_4$ gel, while the pI was 4.3-4.6 and 4.6-5.2 for the diphtheria toxoid and tetanus toxoid, respectively. These broad pI ranges seemed to be caused by the formation of toxoids with various pI values, probably during the formalin-detoxification step. Based on the above isoelectric points, the adsorption experiments were conducted at various pHs,

Table 1. Effect of adsorption pH on degree of adsorption of diphtheria and tetanus toxoids onto freshly preformed aluminum phosphate gel.

Composition	Adsorption pH	Surface charge in adsorption condition		Degree of adsorption (%)
		$AlPO_4$ gel	Toxoid	
50 Lf/ml of diphtheria toxoid and 360 μg Al/ml of $AlPO_4$ gel	5.1	Positive	Negative	100
	5.5	Positive	Negative	98.0
	6.0	Slightly positive	Negative	83.4
	6.6	Neutral	Negative	53.8
15 Lf/ml of tetanus toxoid and 90 μg Al/ml of $AlPO_4$ gel	5.5	Positive	Negative	100
	6.7	Neutral	Negative	63

Table 2. Degree of adsorption of diphtheria toxoid, tetanus toxoid, and HBsAg according to concentration of alum adjuvants.

Antigens	Adjuvant used	Adsorption composition		Degree of adsorption (%)
		Antigens (Lf/ml or μg/ml)	Adjuvant (μg Al/ml)	
Diphtheria toxoid	$AlPO_4$ gel	50 Lf/ml	45	35.8
			90	57.0
			180	87.9
			270	95.0
			360	98.6
Tetanus toxoid	$AlPO_4$ gel	15 Lf/ml	15	76.7
			30	88.8
			60	100
			90	100
HBsAg	$Al(OH)_3$ gel	24 μg/ml	10	80.5
			24	100
			50	100

as shown in Table 1. At pH 5.5, 50 Lf/ml of the diphtheria toxoid was almost completely adsorbed on 360 μg Al/ml of the freshly preformed $AlPO_4$ gel, and 15 Lf/ml of the tetanus toxoid was completely bound on 90 μg Al/ml of the freshly preformed $AlPO_4$ gel. Accordingly, the adsorption of the diphtheria and tetanus toxoids on the freshly preformed $AlPO_4$ gel appeared to be highly pH-dependent.

Adsorption Degree of Diphtheria Toxoid, Tetanus Toxoid, and HBsAg onto Alum Adjuvants

In the case of alum adjuvants, which are known to induce humoral immunity by eliciting Th2-type responses, several studies have confirmed the necessity of excess free alum adjuvants for a higher adjuvant effect [1, 2, 4, 6]. As such, it is very important to determine how much adjuvant is needed to completely adsorb all the antigens. In the current study, a freshly preformed $AlPO_4$ gel was used to adsorb the diphtheria and tetanus toxoids, while a commercial $Al(OH)_3$ gel (Alhydrogel®, Superfos Biosector, Denmark) was used to adsorb the HBsAg. The pH for the adsorption of the diphtheria and tetanus toxoids was fixed at 5.5, and the HBsAg was adsorbed at a pH of 7.0. As a result, the minimal concentrations of adjuvants required for the complete adsorption of the antigens were roughly determined, as shown in Table 2. More than 98% of 50 Lf/ml of the diphtheria toxoid was adsorbed on around 360 μg Al/ml of the $AlPO_4$ gel, 100% of 15 Lf/ml of the tetanus toxoid on about 62 μg Al/ml of the $AlPO_4$ gel, and all 24 μg/ml of HBsAg on about 24 μg Al/ml of the $Al(OH)_3$ gel.

Effect of Age of Aluminum Phosphate Gel on Adsorption of Toxoid

The age of the $AlPO_4$ gel is also known to be an important factor in the adsorption of antigens. Accordingly, in the current study, to determine the correlation between the age of the $AlPO_4$ gel and its adsorptive capacity, the trend of the adsorption degree was investigated with the adsorption of the diphtheria toxoid on preformed $AlPO_4$ gels that were between 0 day (freshly preformed) and 110 days old (Table 3). The adsorptive capacity of the $AlPO_4$ gel continuously decreased with time. Thus, on day 110 after the alum gel preparation, about 15–30% of the initial adsorptive capacity had disappeared, implying that the age of the $AlPO_4$ gel was closely related with the deterioration of its adsorptive capacity. Hence, only a freshly preformed $AlPO_4$ gel was used to prepare the diphtheria toxoid-adsorbed bulk and tetanus toxoid-adsorbed bulk for the combined DTwP-HepB vaccine.

Desorption Degree of Antigens According to pH of Combined DTwP-HepB Vaccine

To formulate five combined DTwP-HepB vaccines (Formulations I, II, III, IV, and V), as shown in Table 4, the

Table 3. Effect of age of preformed aluminum phosphate gel on degree of adsorption of diphtheria toxoid.

Antigen	Age of $AlPO_4$ gel (day)	Degree of adsorption (%)		Adsorptive capacity (Lf/Al)	
		180 μg Al/ml	360 μg Al/ml	180 μg Al/ml	360 μg Al/ml
50 Lf/ml of diphtheria toxoid	0 (freshly preformed)	88.0	98.6	0.244	0.137
	7	76.3	98.0	0.212	0.136
	21	63.3	93.8	0.176	0.130
	110	62.7	83.5	0.174	0.116

Table 4. Degree of desorption of diphtheria toxoid, tetanus toxoid, and HBsAg according to small change of pH in combined DTwP-HepB vaccine.

Combined DTwP-HepB vaccine	Antigen composition				Aluminum phosphate gel (μg Al/ml)	Formulation pH	Degree of desorption (%)		
	D (Lf/ml)	T (Lf/ml)	wP (OU/ml)	HBsAg (μg/ml)			D	T	HBsAg
Formulation I	30	5	20	24	440	6.80	NA[1]	NA[1]	NA[1]
Formulation II	30	5	20	24	800	6.80	24	43	0
Formulation III	30	5	20	24	800	7.10	42	57	0
Formulation IV	40	15	20	24	800	7.10	46	49	0
Formulation V	40	15	20	24	800	7.26	71	75	0

[1]: Not assayed.
D: diphteria toxoid; T: tetanus toxoid; wP: whole cell pertussis.

concentrated adsorption bulks [Diphtheria toxoid-adsorbed bulk: 210 Lf/ml of diphtheria toxoid, 2,520 μg Al/ml of freshly preformed $AlPO_4$ gel, pH 5.5; Tetanus toxoid-adsorbed bulk: 50 Lf/ml of tetanus toxoid, 600 μg Al/ml of freshly preformed $AlPO_4$ gel, pH 5.5; HBsAg-adsorbed bulk: 72 μg/ml of HBsAg, 72 μg Al/ml of $Al(OH)_3$ gel, pH 7.0] and a concentrated detoxified whole cell pertussis bulk (192 OU/ml of whole cell pertussis, pH 7.0) were used. The final aluminum concentration was adjusted by supplementing with the free $AlPO_4$ gel, and the final pH was adjusted within a range of 6.8 to 7.26. The reason for this neutral pH range was because the whole cell pertussis unfortunately aggregated with the diphtheria toxoid-adsorbed bulk in a weakly acidic pH. The degree of adsorption of toxoids and HBsAg was assayed for the above five formulations using a Western blot analysis and the Gel-Pro software mentioned in *Materials and Methods*. As a result, the dissociation of a considerable fraction of the toxoids from the $AlPO_4$ gel was found to be within this neutral pH range, plus the degree of desorption of the toxoids significantly increased, when the combined DTwP-HepB vaccine was in higher pH. At pH 7.26, the degree of desorption of both the diphtheria and tetanus toxoids increased to about 70%, whereas the degree of adsorption of HBsAg remained completely stable. This dissociation of the diphtheria and tetanus toxoids appeared to be due to the electrostatic repulsion between the toxoids and the $AlPO_4$ gel. Therefore, these results show that both toxoids adsorbed under an acidic pH could easily be desorbed in the combined DTwP-HepB formulations at neutral pH range.

The aggregate formation within an acidic pH range around 5.5, which seemed to be caused by the electrostatic attraction between the whole cell pertussis with a negative charge and the diphtheria toxoid-adsorbed bulk with a positive charge (PZC of pH 6.1-6.8), made it impossible to prepare a well-suspended combined DTwP-HepB vaccine. However, in the case of the combined DTwP-HepB vaccines at pH over 6.8, no aggregates were formed even during long-term storage at 4°C.

Immunogenicity of Combined DTwP-HepB Vaccines, and Separately Simultaneous Administration Case Using Combined DTwP Vaccine and Mono HepB Vaccine

The GM anti-HBsAg IgG titer and potency of all the antigens for formulations I, II, III, and IV in Table 4 were investigated (Table 5). For all the formulations, the potencies of all the antigens met with the K-FDA criteria for a combined DTwP vaccine and mono HepB vaccine. The combined DTwP-HepB vaccine with a higher aluminum concentration exhibited a stronger immune response to HBsAg (comparison of formulations I and II), which was in agreement with the previous findings on the necessity of excess alum gel for a higher adjuvant effect [2, 4, 6] and our previous report showing a proportional correlation between the alum gel concentration and the HBsAg immunogenicity in combined DTaP-HepB vaccines [1]. For the combined DTwP-HepB vaccines at different pHs (comparison of formulations II and III), the HBsAg immunogenicity of formulation II was slightly higher at a higher pH. However, the immune response to the diphtheria and tetanus toxoids was lower at pH 7.1 than at pH 6.8, probably because of the low degree of adsorption at pH 7.1 (Gupta and Siber previously reported that immunogenicity in mice to the diphtheria toxoid adsorbed on an $AlPO_4$ gel was dependent on the degree of adsorption [5]). The GM IgG titer for the diphtheria and tetanus toxoids was not measured in the current study, but will be investigated in detail in another study. Formulation IV with a higher DT content induced the highest HBsAg immunogenicity, where the protective anti-HBsAg antibody rate (percentage of the mice above 10 mIU/ml of protective anti-HBsAg IgG titer) was 90% and the GM anti-HBsAg IgG titer was 330 mIU/ml. This corresponds to a previous report by the current authors where a higher DTaP content in a combined DTaP-HepB vaccine was found to induce a stronger immune response to HBsAg [1]. Furthermore, formulation IV not only exhibited a GM anti-HBsAg titer similar to the case of separately simultaneous administration [combined DTwP vaccine (40 Lf/ml of diphtheria toxoid, 15 Lf/ml of tetanus toxoid, 20 OU/ml of detoxified whole

Table 5. Comparison of combined DTwP-HepB vaccines and separately simultaneous administration case in terms of immune response of HBsAg and potency of all antigens.

Vaccines		Immunogenicity					
		HBsAg		Potency[1] of D, T, wP, and HBsAg			
		GM anti-HBsAg IgG titer (mIU/ml)	Protective anti-HBsAg antibody rate (%)	D (Unit/ml)	T (Unit/ml)	wP (Unit/ml)	HBsAg (ED_{50}, μg/ml)
Combined DTwP-HepB vaccine	Formulation I	160	73	2-4	4	14.3	0.44(0.38)[2]
	Formulation II	239	87	2-4	4	16.2	0.40(0.38)[2]
	Formulation III	253	90	2	4	13.4	0.38(0.38)[2]
	Formulation IV	330	90	2	4	12.7	0.34(0.38)[2]
[1]Separately simultaneous administration of DTwP vaccine and HepB vaccine		347	90	2	4	15.9	0.37(0.38)[2]

[1]: Potency satisfies K-FDA criteria (D: ≥2 units/ml; T: ≥2 units/ml; wP: above potency (12 units/ml) of reference whole cell vaccine; HBsAg: statistically equivalent value with ED_{50} of reference HepB vaccine) of combined DTwP vaccine and mono HepB vaccine.

[2]: ED_{50} value (μg/ml) of reference HepB vaccine.

cell pertussis, 300 μg Al/ml of $AlPO_4$ gel, pH 7.1) and mono HepB vaccine (Hepavax®, 24 μg/ml of HBsAg and 500 μg Al/ml of $Al(OH)_3$ gel)], but also satisfied the potency criteria of the K-FDA for all the antigens. For the case of separately simultaneous administration, the protective anti-HBsAg antibody rate and GM anti-HBsAg IgG titer were 90% and 347 mIU/ml, respectively, and the potencies of all the antigens satisfied the K-FDA criteria.

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