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Recombinant Hepatitis B Dry Vaccine Formulation and In Vitro and In Vivo Potency Testing

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Abstract : Hepatitis B vaccines are available in the market in the form of liquid suspensions that is heat sensitive. The study was conducted to produce the hepatitis B vaccine formula which can be managed out of the cold chain.
Utilization of the drying instrument indicates that liquid vaccine formula can be dried and monitored to produce quality vaccines powder. Drying techniques used include: spray drying, freeze drying and vacuum drying. Vaccine formulas were prepared as much as 6 samples with codes F-A through F-F, which reflects the composition of fillers and drying techniques. The powder vaccine was characterized by physical, chemical and antigenic potential as well as an accelerated stability. Vaccine immunogenicity carried out by an ELISA test using a Kit Anti-HBs-Elisa.
The drying technique affecting the decrease of pH and the potential of antigenic vaccine. The combination of trehalose and mannitol did not provide a significant difference to the pH and the relative potency of dried vaccine. The vaccine which was dried by freeze-drying with the composition of trehalose-mannitol (7:3) showed the relative potency in vitro at 97,78% and in vivo at 35,6%.
A dry Hepatitis B Vaccine F-B has an opportunity to be managed out of the cold chain system.
Keywords : Hepatitis B Surface Antigen (HBsAg); in vitro; in vivo; relative potency; vaccine.

Introduction

Hepatitis B vaccine is a pharmaceutical preparation which is sensitive to heating and freezing. Exposure to excessive heat can lead to decreased stability and shelf life of the vaccine, otherwise freezing the vaccine can cause permanent loss of potency (Diminsky, Moav, Gorecki, & Barenholz, 1999). To ensure the stability and potency during storage and distribution, the vaccine is kept at a temperature 2^0C - 8^0C known as the cold chain system.

Indonesia, which has a tropical climate, is a challenge in the management of the stocks of vaccines for the liquid preparation is not resistant to heating. Dried vaccine formulation may improve the stability of the preparation and keep the potential reduction of antigen caused by chemical and physical degradation such as hydrolysis, precipitation, clotting or fluctuations in pH (Saluja et al., 2010). Some drying techniques have been applied in the preparation of vaccines, among others: freeze drying, spray drying and vacuum drying.

In the dried vaccine, antigen molecule movement is much smaller when compared with the liquid vaccine as a result, protein damage will be slower. However, during the drying process, protein will be degraded and may lose its potency. Class saccharide compounds such as mannitol, sucrose, trehalose and lactose, has been widely used as a stabilizer to prevent the degradation of proteins during the process of drying and storage (Tonnis et al., 2014).

Recombinant vaccine antigens are generally weak and often fail to provide the expected immune response that should be added adjuvant. Adjuvants commonly used in vaccines are alum salts such as Al (OH)x(PO4)y and KAl(SO4)2.12H2O and Al(OH)3. The use of aluminum hydroxide is preferred because it is proven safe, stable and commonly used in vaccine (Vecchi, Bufali, Skibinski, O'hagan, & Singh, 2012).

Stability during storage and distribution of vaccines will affect the potential immunogenicity (Tonnis et al., 2014). To determine the stability of the vaccine at its storage temperature it needs to be accelerated stability test that the time required for a shorter observation.

Experimental

Materials

The active ingredient in the form of bulk antigen derived from Biofarma, Co. Ltd. with a concentration of 0.9445 mg/ml HBsAg in Phosphate buffered saline (PBS). Comparator vaccine using a liquid hepatitis B vaccine recombinant (HBV), Biofarma, Co. Ltd. packaging 0.5 mL Prefilled Injection Device Batch No. 3653815.

All excipient used in this study were given from Hexpharm, Co. Ltd and Dwipar L.A. Co. Ltd. with the pharmaceutical grade or higher. The excipient included Trehalose, mannitol, polysorbate 80 and dried gel Aluminum hydroxide (Al(OH)3) as an adjuvant.

Antigen-adjuvant adsorption

Antigen-adjuvant adsorption performed by incubating adjuvant with antigen in optimal conditions. The process is continuous at pH 6.8 -7.2 and a temperature of 2-8 ° C and the stirring speed of 40-60 rpm (Awate, Babiuk, & Mutwiri, 2013). The mixture is stirred at least for two hours to obtain suspension of antigen-adjuvant.

Formulation

The vaccine formulation made by mixing the excipients in phosphate buffer with the suspension of antigen - adjuvant. Samples are prepared in six formulas and each formula containing 20 mcg / mL HBsAg adsorbed on 0.5 mg of adjuvant Al3+ with a ratio of carrier material trehalose: mannitol (1:1 and 7:3) and polysorbate 80 (0,05%). The vaccine formulation is done at a temperature of about 4°C with the stirring speed at 40-60 rpm (Awate et al., 2013).

Spray drying (SD)

The liquid vaccine formulation was sprayed using Spray Dryer SD 1000, Eyela Corp., Japan. The Spray-drying conditions were as follows at feed rate 10 mL/min, inlet temperature at 130 ° C and its resulting outlet temperature between 80°C - 84°C. The resulting powder is maintained so as not exposed to temperatures above 45 ° C and placed in a vacuum desiccator with silica gel dryer at room temperature (Chen et al., 2010).

Freeze drying (FD)

The liquid vaccine formulation stored at freeze drying tube and dried using FDU Freeze Dryer 1200, Eyela Corp., Japan. The condition run at temperature adsorption at -40 ° C. vacuum pressure of 9.5 Pa and at least 20 hours of drying time (Li, Thakkar, Ruwona, Williams, & Cui, 2015).

Vacuum drying (VD)

The sample is introduced into a glass beaker and placed into Vacuum Dryer VOS 301 SD, Eyela Corp., Japan. The optimization of this method can be done with the temperature of the tool 25 ° C and slowly increased to 30 °C, vacuum pressure at 3.15 kPa and 24 hours of drying time (Jangle & Pisal, n.d.).

Sterilization

All prepare formulation was sterilized by 27,5 kGy Gamma Irradiation at Natural Rubber Irradiator of National Atomic Energy Agency.

Moisture content

Briefly, testing for moisture content using a Karl Fisher Coulometer (Model 737) equipped with a drying oven (Model 707).

pH measurement

pH measurements made after the preparation of the vaccine is reconstituted with aqua pro injection using a pH meter (Eutech Instrument Type-510, Singapore). Observation pH should be done before In vitro evaluation of each month.

Antigenicity of HBsAg

The potency of vaccines was determined by a quantitative enzyme immune assay using an enzyme immunoassay(EIA) kit (Murex ® monoclonal diagnostic kit Version 3.0, Abbot lab.IL.). Each sample was examined at four different dilutions with three repetitions against a linear fitting to the responses of control standard dilutions. The potency was expressed as the quantity of HBsAg (μg)

Immunogenicity test

Testing is carried out on the vaccine formula which indicates a highest and lowest In vitro potency. Four to five-weeks-old deutch democratic Yokohama (ddY) mice were used to assess the immunogenicity of two formulas. Vaccines were reconstituted in saline and injected into animals as much as 1 ml by Intra Peritoneal (IP) injection using a 1 ml disposable syringe with a needle size 26 G x ½ ". Injection process starting from the smallest dose dilution (1: 256). Blood was collected via heart bleeding 28 days after the final immunization and the antibody responses were analyzed using Anti-HBs-Elisa kit (ETI-AB-AUK 3, Diasorin®, SPA).

Stability test

Samples are stored in the oven (Memmert®, Germany), with a temperature of 40^0C ± 2 ^{0}C/ 75% RH during testing range. At the end of each month evaluation, vaccine tested in vitro antigenicity and pH. Samples that have been tested are not returned to the oven. Testing carried out during the first three months ("ICH Q5C - Stability testing of Biotechnological / Biological products 1," n.d.).

Results and Discussion

Table1: Summary of all formulas

Formula	Drying methods	Excipient ratio (T:M:P)	Moisture content (%)	pH	Potency Recovery (%)	Vaccine sterility
F-A	Freeze drying	1:1: 0,05	3,91	6.82	96,427	Sterile
F-B	Freeze drying	7:3: 0,05	3,77	6,82	97,780	Sterile
F-C	Vacuum drying	1:1: 0,05	7,12	6,84	92,953	Sterile
F-D	Vacuum drying	7:3: 0,05	6,24	6,84	93,970	Sterile
F-E	Spray drying	1:1: 0,05	1,94	6,78	61,192	Sterile
F-F	Spray drying	7:3: 0,05	2,18	6,78	64,829	Sterile
Bulk				6.70	109,319	
HBV				6,81	100	

Notes: T: Trehalose; M: Mannitol; P: Polysorbate 80, HBV: Hepatitis B Vaccine from Biofarma Co. Ltd.

Table 2: Stability test result

Formulation	Month	pH	Potency recovery (%)
F-A	1st	6,80	89,38
	2nd	6,78	82,42
	3rd	6,77	72,83
F-B	1st	6,80	91,99
	2nd	6,79	84,02
	3rd	6,78	74,12
F-C	1st	6,80	83,53
	2nd	6,76	78,10
	3rd	6,73	67,74
F-D	1st	6,81	85,20
	2nd	6,77	80,89
	3rd	6,73	69,30
F-E	1st	6,79	
	2nd	6,78	
	3rd	6,78	
F-F	1st	6,79	
	2nd	6,78	
	3rd	6,78	
HBV	1st	6,77	90,45
	2nd	6,72	58,72
	3rd	6,65	17,03

Antigen-adjuvant adsorption

The antigen-adjuvant suspension produced during pre-formulation shows relatively clear liquid and colorless. This is due to the dry gel Al $(OH)_3$ is dissolved in a solution of 0.9% Na Cl and HBsAg bulk suspension is relatively clear and colorless. Bulk antigen very easily dispersed in a solution of 0.9% Na Cl physiological because it does not contain a grain of water-insoluble particles.

Formulation

The resulting vaccine suspension of all formulas is relatively clear and colorless. This is caused by all the excipients used is a material that is readily soluble in water. Excipients used is a material commonly used in parenteral preparations as well known to have a physical and biochemical stability suitable for powder formulations.

The whole vaccines contain trehalose in two different compositions. The use of trehalose, other than as a bulk-forming material is also known to have properties as a protein stabilizer. It is important to prevent the degradation of antigens during the drying process (Chang & Pikal, 2009). The use of trehalose together with mannitol as a bulk-forming material provides better powder texture. In addition, trehalose and mannitol reducing properties were lower, thereby reducing the possibility of reaction with protein (Maa et al., 2007).

Moisture content

Measurements of water content in the dry vaccine aim to measure the humidity will affect the flowability of powders and chemical stability during the storage process. The test results showed that F-E and F-F have a moisture content that is relatively low compared to other formulas. This is because the primary method, droplets of the vaccine was warming up to 130 ° C so that the dehydration process runs evenly and quickly in the whole particle (Li et al., 2015). While the F-C and F-D contain relatively high levels of water due to evaporation of water is not going well.

In vitro study result

Formula F-C and F-D were dried VD showed a decrease in potency when compared with the potential of bulk antigen but still meet the 95% confidence limit. While the vaccine F-E and F-F were dried in SD showed significant differences in potency when compared with the HBsAg bulk. This condition occurs because the vaccine was under pressure during the drying mainly on the process of atomization and drying droplet at high temperature (Li et al., 2015). This condition causes the loss of potential antigens significantly during the drying process.

Formula F-A and F-B were dried FD showed a higher in potency than others and meet the 95% confidence limit. A freeze-drying technique produces a porous dry cake in a tube and an ideal moisture content in the final powder. Water evaporate during the primary drying massively and after secondary drying crystals water also eliminate (Adams, Cook, & Ward, 2015). However, the antigenicity of HBsAg decreases during freezing.

In vivo assay

Blood of the test animals was collected by the technique bleeding heart. This technique is selected because it can produce more blood volume and then do centrifugation at 10,000 rpm with 4^0C temperature for 10 minutes to obtain serum containing antibodies. Serum produced from each of the animal tests ranged from 0.3 to 0.5 ml and stored in a refrigerator at a temperature of 2-8 ° C prior to further testing.

The results showed that the relative potency of dried vaccine F-B by 35,6.0% while the F-E was 11%. In the F-E, spray drying technique extremely reduce antigenicity of vaccine as a result, the vaccine did not show enough potential during both in vitro and in vivo assays.

According to the British Pharmacopoeia appendix xiv k vol.5, 2013, stated that the 95% confidence limit for In vivo test is obtained if the test results of samples are in the range of 30-300%. From the test results obtained by the relative potency, F-B was 35,6% and meet the test requirements.

Accelerated stability test

Accelerated stability testing was conducted to predict the stability of the preparation after a certain period and it is important to ensure the shelf life of the vaccines. During the testing range, dried vaccine experienced a slight discoloration on the powder to become paler. In the F-C and F-D, the consistency of the powder becomes denser but are destroyed when given vibration in the vial. Compaction of the powder occurs because the water content decreases due to heating (Abdul-Fattah et al., 2007).

The liquid suspension HBV vaccine tends to decrease of pH. The high water content in the liquid vaccine causes the heat retained in the medium in which the water is able to absorb and hold heat well (Abdul-Fattah et al., 2007). Increased temperatures lead to hydrolysis of proteins and vaccine component changes and the emergence of clotting preparations (Katdare, Chaubal, & London, n.d.). It can be observed visually by a color change from relatively clear dosage be inclined cloudy**.**

Heat stability of the liquid vaccine is strongly influenced by the pH, degradation of the stocks, both chemical and physical, are influenced by changes in pH. Chemical degradation generally involves the catalyzed reaction such as acid-base hydrolysis of peptide bonds of proteins. Degradation in physics emerged as a result of changes in protein content and adjuvant (Katdare et al., n.d.). So as to prevent the depletion of the liquid vaccine that must be managed in a cold chain system.

The pH profile showed that the dried vaccine formulas were maintaining stability over a range of pH testing. The small water content in the dried vaccine prevents a reaction catalyzed acid-base. The use of phosphate buffer effectively kept the pH changes due to heating (Katdare et al., n.d.) . This can be observed from the entire formula showed a decrease in pH were not significant when compared with the liquid vaccine.

The whole dried vaccine formulas show pH profiles are likely to be stable during the test but were unable to sustain the potential antigenic HBsAg. The Stable pH profile and the potential antigenic HBsAg was

not decreased sharply indicates that the dry Hepatitis B vaccine has the prospect to be managed at room temperature.

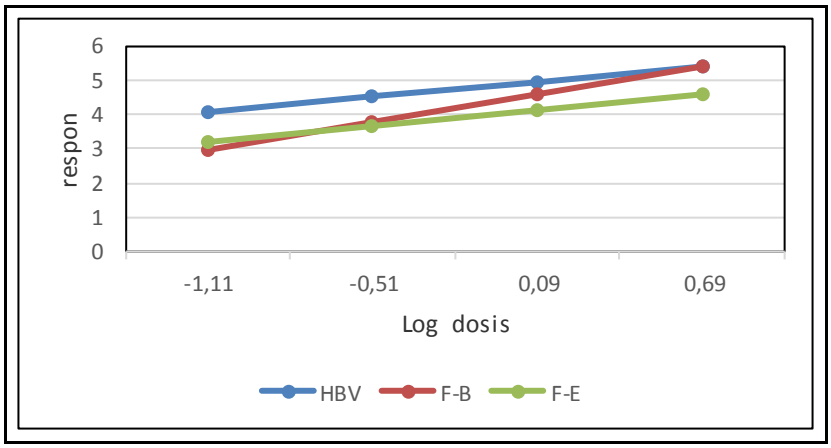

Fig. 1: Immune respond after 28th day vaccine injected in mice

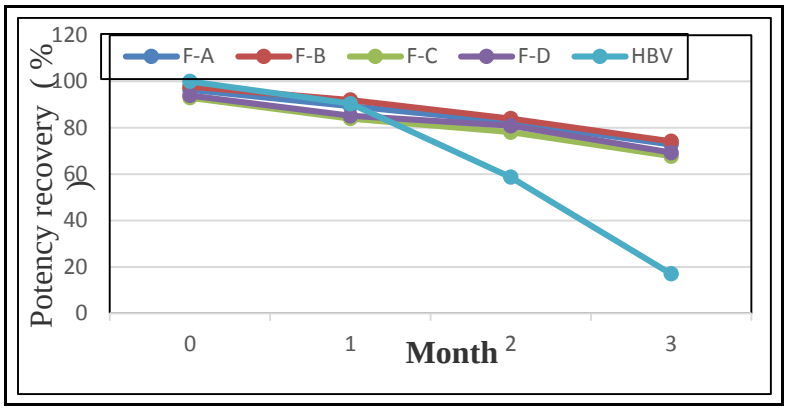

Fig. 2: Formulation screening for the biochemical stability of four dry vaccines. HBsAg potency at each time-point was analyzed by enzyme immunoassay(EIA) kit(Murex ® monoclonal diagnostic kit Version 3.0, Abbot lab.IL.

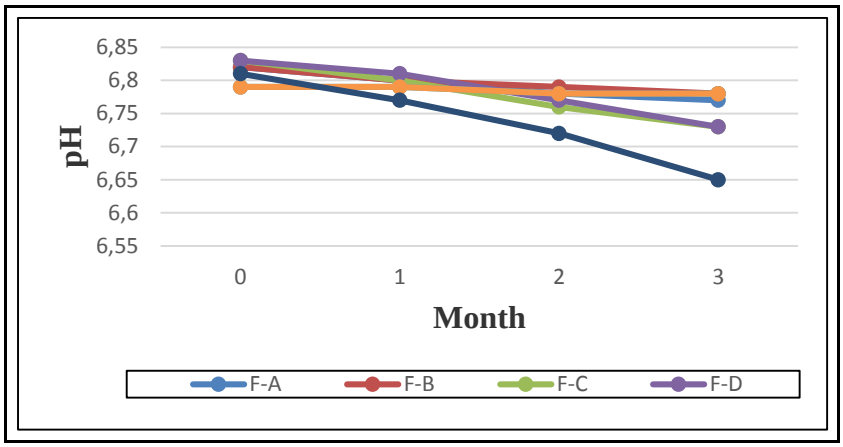

Fig. 3: pH changes during the three months of observation

Table 3. In vivo potential test in mice.

Dosis	HBV		F-B		F-E		Control	
	n	**r**	**n**	**r**	**n**	**r**	**n**	**r**
5	6	4	6	3	6	2	6	0
1,25	6	3	6	2	6	1		
0,3125	6	2	6	2	6	1		
0,07815	6	1	6	1	6	0		
Lower limit			0,02		0,002			
Upper limit			3,481		0,86			
Potency			0,356		0,11			

Notes : n = Samples r = Reactive samples

Conclusion

Differences in the composition of bulking agents did not show any significant differences to the pH and potential antigenic vaccine. Drying technique effect on the decline in potential vaccine antigen in which the freeze-dried vaccine with the potential to produce relatively high at 97,8% with a combination of trehalose: mannitol (7:3). In vivo testing indicate potential immunogenic F-B at 35.8% and has an opportunity to be managed out of cold chain system.

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