

Research Paper

A heat-stable hepatitis B vaccine formulation

Jan Jezek,^{1,*} Dexiang Chen,² Lynne Watson,¹ Jill Crawford,¹ Shalimar Perkins,² Anil Tyagi³ and LaToya Jones-Braun³

¹Arecor Limited; Sharnbrook, Bedfordshire UK; ²PATH; Seattle, WA USA; ³University of Colorado, Denver; Aurora, CO USA

Abbreviations: HBsAg, hepatitis B surface antigen

Key words: hepatitis B, vaccine, formulation, stable, aluminum hydroxide adjuvant

The purpose of the present study was to develop a formulation of recombinant hepatitis B vaccine with improved stability at elevated temperatures. A validated *in vitro* antigen reactivity assay was used to measure the stability of the vaccine. The formulation development focused on modification of the interactions between the antigen and aluminum hydroxide adjuvant and subsequent optimization of the ionic aqueous environment of the adsorbed vaccine. A formulation of hepatitis B vaccine containing 40 mM histidine and 40 mM phosphate at pH 5.2 had considerably improved stability at elevated temperatures as measured by the *in vitro* antigen reactivity assay. The formulation exhibited 9-week stability at 55°C and was subsequently shown to be stable both at 37°C and at 45°C for at least 6 months based on the *in vitro* antigen reactivity and immunogenicity in mice. The formulation comprises only excipients which have a history of safe use in approved drug products. The new vaccine formulation has the potential to be used outside the cold chain for part of its shelf life. This may improve the immunization coverage, simplify the logistics for outreach immunization, and ensure the potency of the vaccine in areas where the cold chain is insufficient.

Introduction

All existing vaccines currently used in immunization programs are heat sensitive and require a cold chain for transportation and storage. Maintaining the cold chain at a specific temperature range, normally 2°C to 8°C, is both costly and challenging for resource-constrained countries. The temperature sensitivity of vaccines creates complex logistics for immunization programs trying to reach rural populations and in certain cases limits immunization coverage. The unreliability and breakage of the cold chain in the periphery may result in vaccine damage or wastage not only in developing countries, but also in places with established cold chains where spikes in temperature may occur due to various

interruptions. These problems can be partially or wholly alleviated if vaccines are stable at extreme temperatures.

Hepatitis B vaccine is one of the most heat-stable children's vaccines, maintaining stability for up to four years at temperatures between 2°C and 8°C. A 50 percent loss of potency of the vaccine has been reported to be observed after 9 months at 20°C to 26°C, after one month at 36°C to 40°C and after three days at 45°C.¹ By taking advantage of the relative thermostability of hepatitis B vaccine, several countries, including Vietnam, Indonesia and China, have been experimenting with out-of-cold chain use for immunizing infants with the birth dose. Vaccines are kept by midwives or clinics without refrigeration for up to one month and administered to infants shortly after birth. Results have shown that this out-of-cold chain use has improved the coverage of the birth cohort without compromising the effectiveness of the vaccine.²⁻⁵ Improvements to the heat stability of hepatitis B vaccine would further ease immunization logistics and allow even wider and safer immunization coverage, especially in tropical climates.

Hepatitis B vaccine is a liquid suspension consisting of purified recombinant hepatitis B surface antigen (HBsAg) adsorbed onto aluminum hydroxide adjuvant. HBsAg is a recombinant non-glycosylated lipoprotein complex, which is the primary viral envelope protein responsible for immunogenicity and immunity from hepatitis B virus infection. The recombinant HBsAg polypeptide has a molecular weight of 24 kDa, and self-assembles readily in solution to form liposome-like 22 nm particles presenting antigenic HBsAg epitopes on their surface.⁶ HBsAg has an isoelectric point of approximately 4.5, while aluminum hydroxide adjuvant has a point of zero charge of 11.4. Consequently, electrostatic adsorption is conceivable between the adjuvant and the antigen at a wide range of pH at which the HBsAg is negatively charged and aluminum hydroxide adjuvant is positively charged. The adsorption of HBsAg by aluminum hydroxide adjuvant was shown to exhibit a high affinity adsorption isotherm.⁷ However, in spite of the favorable electrostatic properties, the adsorption of HBsAg by aluminum hydroxide adjuvant was shown to be predominantly due to ligand exchange between the phospholipids of HBsAg and surface hydroxyls of aluminum hydroxide adjuvant.⁷ Electrostatic interactions and hydrophobic interactions have been shown to play a considerably less important role.

*Correspondence to: Jan Jezek; Arecor Limited; Sharnbrook, Bedfordshire MK44 1LQ UK; Email: jan.jezek@arecor.com

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The surface characteristics of the adjuvant particle can be modified considerably in the presence of phosphate anions due to a strong affinity of phosphate to the aluminum cation and consequent ligand exchange between the phosphate anion and hydroxide anion.⁸ Such modification lowers the point of zero charge of the adjuvant causing the particle surface to be less positively charged at a given pH. Consequently, the adsorption of anions from the bulk solution to form the Stern layer surrounding the particle is suppressed. It has been suggested previously⁹ that this effect results in changes in pH microenvironment at the surface of adjuvant particle due to lower adsorption of the hydroxide anion. The presence of phosphate anion thus has a potentially profound effect on the interactions between the antigen adsorbed on an aluminum hydroxide adjuvant particle and its surrounding environment. It is therefore likely that the pH-related interactions affecting the stability of an antigen adsorbed on aluminum hydroxide adjuvant can be controlled by adjusting the level of phosphate in the formulation.

Based on the nature of hepatitis B antigen and its interactions with aluminum hydroxide adjuvant, it appears possible to further improve the stability of the vaccine through changes in formulation. This can be achieved both by modifying the interaction between the recombinant antigen and the adjuvant and by further formulation optimization ensuring native conformation of the antigen is maintained. The purpose of the work reported here was to develop a formulation of hepatitis B vaccine with improved stability at elevated temperatures.

Results

The initial formulation development was carried out by subjecting soluble (neat) and adjuvant-adsorbed HBsAg to heat-stress at 55°C for nine weeks to allow rapid identification of stability trends. The stability of neat HBsAg was shown to be optimal at pH around 8 in a histidine/TRIS buffer mixture (Fig. 1). However, no such trend was observed when studying the effect of pH on adsorbed HBsAg. The stability of the adsorbed antigen was very low across the whole pH range between 5 and 9, showing a marginally better recovery following the heat-stress at the lower end of the pH range. This indicated a destabilizing effect of aluminum hydroxide adjuvant on HBsAg.

The subsequent effort focused on investigation of the effect of different concentrations of phosphate anion on the stability of HBsAg adsorbed on aluminum hydroxide adjuvant. The effect was investigated across a pH range between 4.6 and 7.0, the lower end of this range being deemed an approximate limit of pH acceptability for a vaccine used in humans. Apart from phosphate, the formulations did not contain any other excipients. For the phosphate-free control, histidine (20 mM) was used as a buffer. The stability of adsorbed HBsAg was shown to be strongly dependent on the concentration of phosphate in the formulation (Fig. 2). Less than 20 percent of the original antigenic activity could be recovered after two weeks and less than 8 percent after nine weeks of incubation at 55°C in the phosphate-free histidine buffer across the pH range studied. In contrast, more than 50 percent of the original antigenic activity was recovered after incubation of the vaccine in 20 mM phosphate at 55°C for two weeks and more

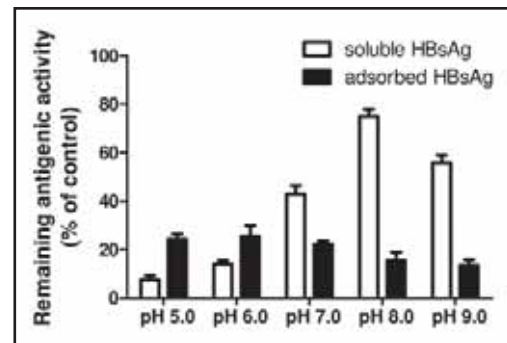


Figure 1. Effect of pH on stability of HBsAg (20 µg/mL) at 55°C for two weeks, both free in solution and using the adsorbed vaccine (Shanvac-B). The data is expressed as a percentage of the control material stored at 4°C. Mixture of 20 mM histidine and 20 mM TRIS was used as a buffer.

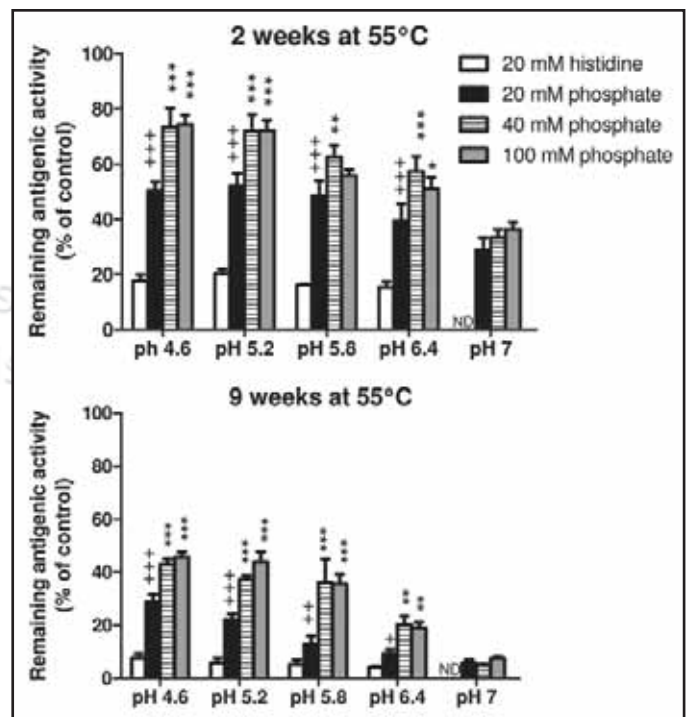


Figure 2. Effect of phosphate anion on the recovery of antigenic activity of hepatitis B vaccine at pH 4.6 to 7.0 following incubation at 55°C for two weeks and nine weeks. The data is expressed as a percentage of the control vaccine, the original Shanvac-B stored at 4°C. The test formulations contained 20 mM histidine, 20 mM phosphate, 40 mM phosphate or 100 mM phosphate. (ND: The histidine formulation was not tested at pH 7.) Key for statistical analysis: 20 mM phosphate vs. 20 mM histidine: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; 40 and 100 mM phosphate vs. 20 mM phosphate: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

than 20 percent after incubation for nine weeks. These were very significant improvements ($p < 0.001$) over the phosphate-free formulation across the pH range 4.6–6.4. The stability was further improved in the presence of higher concentrations of phosphate leading to recovery of antigenic activity of greater than 70 percent after two weeks and greater than 40 percent after nine weeks at 55°C in the presence of either 40 mM or 100 mM phosphate.

Table 1 Effect of phosphate anion and sodium chloride on the adsorption of HBsAg onto aluminum hydroxide antigen

Formulation	Fresh sample (% ± st. dev.)	Following nine weeks at 55°C (% ± st. dev.)
Original Shanvac-B (pH 7.0)	97.1 ± 1.0	96.9 ± 0.6
40 mM phosphate (pH 5.2)	96.8 ± 0.3	97.9 ± 0.7
40 mM phosphate (pH 5.2) + NaCl (150 mM)	98.1 ± 0.4	96.8 ± 1.1
40 mM phosphate (pH 5.2) + NaCl (500 mM)	95.1 ± 0.9	93.9 ± 1.7

The % adsorption is expressed as $(T-S) \times 100/T$ where T is the HBsAg activity in the whole vaccine and S is the HBsAg activity measured in the supernatant.

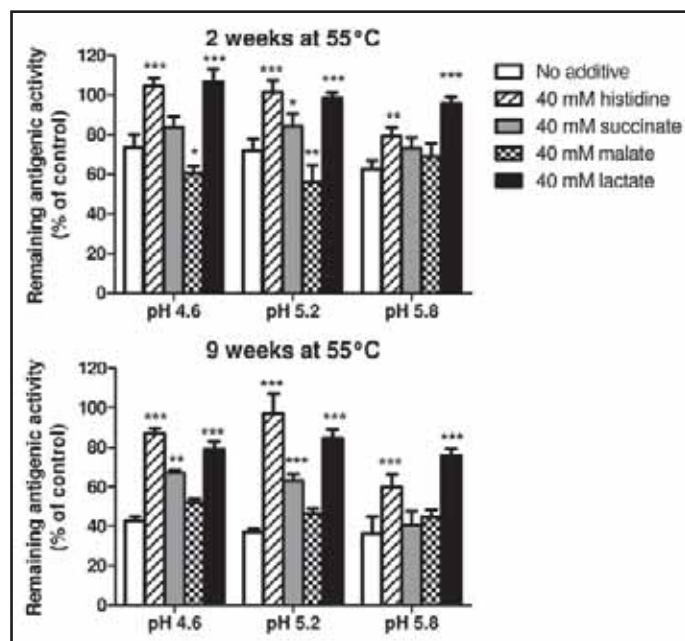


Figure 3. Effect of selected buffering species on the recovery of antigenic activity of hepatitis B vaccine at pH 4.6 to 5.8 in the presence of 40 mM phosphate following incubation at 55°C for two weeks and nine weeks. The data is expressed as a percentage of the control vaccine, the original Shanvac-B stored at 4°C. The additional buffering species tested were 40 mM histidine, 40 mM succinate, 40 mM malate and 40 mM lactate. Key for statistical analysis: additional buffering species vs. no additive *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

The difference in stability of HBsAg in 20 mM phosphate and 40 mM phosphate was again significant ($p < 0.001$ to $p < 0.01$) across the pH range 4.6–6.4. The stability appeared to be best in the pH range of 4.6 to 5.8, and no marked difference in the stability of HBsAg was observed between formulations containing either 40 mM or 100 mM phosphate across the pH range examined ($p > 0.05$). The stability at pH 6.4 and especially at pH 7.0 was considerably lower regardless of phosphate concentration (Fig. 2), so no experiments were carried out at a more alkaline pH.

The initial investigation of the effect of phosphate anion thus indicated two formulation parameters which are essential for ensuring the stability of HBsAg adsorbed on aluminum hydroxide adjuvant: (1) presence of at least 40 mM phosphate anion and (2) pH around 5.2. The adsorption of HBsAg onto the adjuvant did not appear to be adversely affected by the presence of 40 mM phosphate anion at pH 5.2 (Table 1). Furthermore, the presence of 150 mM NaCl in the 40 mM phosphate formulation had no effect of the adsorbed antigen, and only a limited effect was observed when the NaCl concentration was increased to 500 mM. The adsorption characteristics were not changed in any of these formulations following incubation at 55°C for nine weeks (Table 1).

The effect of additional ionic excipients on the stability of HBsAg was investigated in the presence of 40 mM phosphate at pH 4.6, 5.2 and 5.8, covering the optimal conditions discovered during the initial formulation development. Ionic excipients were selected to improve the buffering capacity of the formulation as the buffering capacity of phosphate anion is very limited at a pH around 5.2. The presence of malate anion (40 mM) did not improve the stability of HBsAg at 55°C over that achieved in the presence of phosphate anion only. The presence of succinate anion had a small beneficial effect on the HBsAg stability resulting in greater than 60 percent recovery of the antigenic activity following incubation at 55°C for nine weeks at pH 4.6 and 5.2. In contrast, the presence of lactate anion and particularly the presence of histidine resulted in considerably improved stability of HBsAg ($p < 0.001$ across the pH range 4.6–5.8, Fig. 3). More than 85 percent of the antigenic activity was recovered following incubation at 55°C for nine weeks in the presence of lactate and more than 90 percent in the presence of histidine at pH 5.2. This pH appeared to be optimal, especially if histidine was used as the additional excipient. The stability was poorer at pH 5.8, but slightly better in the presence of lactate than in the presence of histidine.

No significant antigenic activity was measured in the supernatants of the formulations based on phosphate (40 mM) and either histidine (40 mM) or lactate (40 mM) at pH 5.2. This suggested that the HBsAg remains bound to the adjuvant in the presence of these excipients (Table 2).

Lead candidate formulations which showed best stability under the heat-stress at 55°C, comprising histidine (40 mM) and phosphate (40 mM) at pH 5.2, were subsequently evaluated in stability studies at 45°C and 37°C. The in vitro stability of the reformulated vaccine was compared with that in the original sample of Shanvac-B. The antigenic activity of the Shanvac-B vaccine dropped to approximately 60 percent after one month at 37°C and about 30 percent after 6 months at 37°C. Similarly, the activity following incubation at 45°C dropped to about 40 percent after one month and less than 10 percent after 6 months (Fig. 4). In contrast, the antigenic activity in the formulations based on histidine and phosphate remained at greater than 80 percent of the original level after incubation at 45°C for 6 months and virtually at 100 percent after incubation for 6 months at 37°C. The stabilizing effect was not compromised by the presence of 100 mM sodium chloride at 37°C (Fig. 4). The effect of sodium chloride was not studied at 45°C.

The stabilized formulation consisting of histidine (40 mM) and phosphate (40 mM) at pH 5.2 was subsequently tested in mice. Relative potency was compared between the original formulation of hepatitis B maintained either at 4°C or at 37°C for 6 months and the stabilized formulation treated under the same conditions (Table 3). Treatment of the original vaccine at 37°C for 6 months caused a small decrease in the potency relative to that of the vaccine stored at 4°C although the change was not statistically significant. In contrast, the stabilized vaccine, following 6 month heat stress at 37°C, showed no indication of potency change when compared to the 4°C control.

The *in vivo* immunogenicity of hepatitis B vaccine is dependent on both the stability of HBsAg and the particulate structure of the aluminum hydroxide adjuvant. It has been suggested¹ that agglomeration of adjuvant particles affects the immunogenicity of vaccines and the size distribution of the adjuvant particles is therefore an important indicator of the aluminum salt adjuvant activity. Pairing the Coulter Counter results with *in vitro* and *in vivo* activities of the hepatitis B vaccine following various freeze-thaw treatments, we also observed this correlation.¹⁰ In the current study, the measured particle size distribution in the original vaccine formulation was as expected, >99% of the particles within the 1.5 to 3 micron range. Importantly, the change of the vaccine formulation did not appear to have an effect on the size distribution of vaccine particles (Table 4). The distribution of the particle size was comparable between the original Shanvac-B formulation and the stabilized formulation based on histidine and phosphate at pH 5.2.

Discussion

All currently marketed formulations of hepatitis B vaccine are intended for storage at 2°C to 8°C. These are typically liquid suspensions comprised of an aluminum hydroxide gel adjuvant and approximately 18 mM phosphate buffer adjusted to pH 7.0 ± 0.1.⁶ Consequently, there is a certain degree of surface modification of the adjuvant in these formulations due to ligand exchange between surface hydroxides and the phosphate anion from the buffer.⁸ Surface modifications of aluminum hydroxide adjuvants by adding phosphate anions prior to adsorption of the antigens has recently been reported to improve the immune response to α casein and HbsAg by reducing the strength of the adsorption to the adjuvant.^{11,12} Such surface modification results in changes in pH microenvironment at the surface of the adjuvant particle, which we suggest are very likely to affect the stability of the antigen adsorbed on the adjuvant surface.

The degree of the surface modification of adjuvant particles by phosphate has been reported to be proportional to the phosphate concentration in the formulation.¹³ The pH optimum for stability of HBsAg dissolved in aqueous solution was found to be around 8, with apparent lower antigenic activity in more acidic

Table 2 Effect of histidine and lactate anion on the adsorption of HBsAg onto aluminum hydroxide antigen in the presence of phosphate

Formulation	Fresh sample (% ± st. dev.)	Following nine weeks at 55°C (% ± st. dev.)
Original Shanvac-B (pH 7.0)	96.8 ± 0.8	95.1 ± 1.9
40 mM phosphate + 40 mM histidine (pH 5.2)	97.4 ± 0.8	97.2 ± 1.0
40 mM phosphate + 40 mM lactate (pH 5.2)	95.6 ± 1.5	96.9 ± 1.1

The % adsorption is expressed as $(T-S) \times 100/T$ where T is the HBsAg activity in the whole vaccine and S is the HBsAg activity measured in the supernatant.

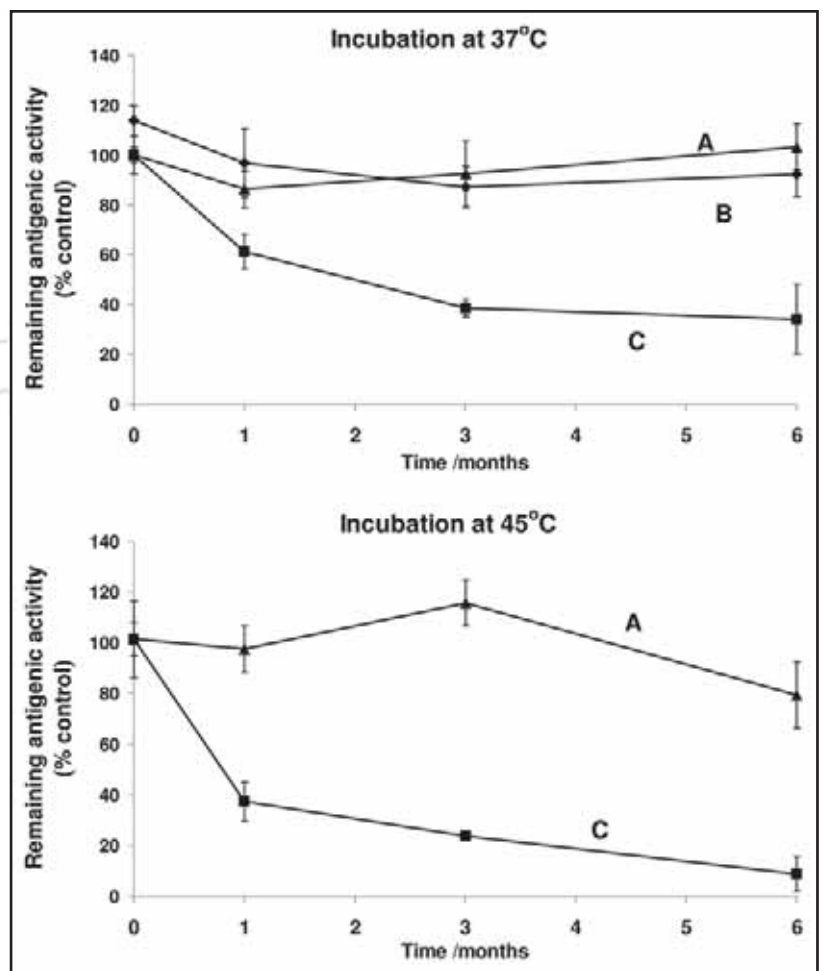


Figure 4. Recovery of antigenic activity of hepatitis B vaccine formulations at 37°C and at 45°C. The formulations were: (A) 40 mM phosphate + 40 mM histidine, pH 5.2; (B) 40 mM phosphate + 40 mM histidine + 100 mM NaCl, pH 5.2; and (C) original Shanvac-B formulation. Formulation (B) was not tested at 45°C.

or alkaline pH solutions. Moreover, if HBsAg was adsorbed onto aluminum hydroxide adjuvant in the absence of phosphate anions the stability of the antigen was very poor across the pH range between 5 and 9, showing only a marginally better stability at the

Table 3 Relative in vivo potency of the original Shanvac-B vaccine and the stabilized vaccine formulation following incubation at 4°C and 37°C for six months

Sample	Relative potency	
	Average	CI (95%)
Phosphate (40 mM) + histidine (40 mM), pH 5.2 (4°C)	1.00	N/A
Phosphate (40 mM) + histidine (40 mM), pH 5.2 (37°C)	1.11	0.43–2.87
Original Shanvac-B (4°C)	1.13	0.45–2.91
Original Shanvac-B (37°C)	0.86	0.33–2.2

Table 4 Size distribution of aluminum hydroxide adjuvant particles with adsorbed HBsAg antigen in the Shanvac-B vaccine and in the reformulated vaccine containing histidine and phosphate

Sample	Percentage of particles in each size range (μm)			
	1.5–3.0 μm	3.0–6.0 μm	6.0–9.0 μm	>9.0 μm
Original Shanvac-B (pH 7.0)	99.80%	0.19%	0.01%	0.00%
Phosphate (40 mM) + histidine (40 mM), pH 5.2	99.87%	0.12%	0.01%	0.00%

acidic end of this pH range. The destabilizing effect of aluminum hydroxide adjuvant may be due to the previously reported alkaline pH microenvironment at the adjuvant surface.⁹ In this Hem and colleagues study, the adsorbed antigen behaved as though the pH was approximately two units higher than that of the bulk solution. It was demonstrated in the present study that maintaining the in vitro antigenic reactivity of the adsorbed HBsAg stored at elevated temperatures was strongly dependent on the presence and concentration of phosphate in the formulation up to about 40 mM, with no apparent improvement at higher phosphate concentrations.

It was also established that the neutral pH of the currently marketed hepatitis B vaccine is not optimal with respect to the stability of the adsorbed HBsAg antigen in the presence of phosphate. A slightly acidic pH resulted in markedly improved vaccine stability. Thus, the stability of the HBsAg adsorbed onto the aluminum hydroxide adjuvant can be considerably improved by formulating in the presence of 40 mM phosphate at a pH around 5.2. No measurements of the surface charge of the aluminum hydroxide adjuvant were carried out in this study, but it is believed that the main function of phosphate is in modification of the surface charge by ligand exchange, as described previously^{8,9} resulting in a pH microenvironment at the particle surface optimal for stability of adsorbed HBsAg in a suspension maintained at pH around 5.2.

The surface modification of the adjuvant particle by 40 mM phosphate together with the changed pH of the formulation may

raise concerns about the efficient adsorption of the antigen onto the adjuvant particle. However, the in vitro activity of the adsorbed HBsAg did not appear to be adversely affected by the presence of 40 mM phosphate anion at pH 5.2 and was comparable to that in the currently marketed vaccine preparation. This agrees well with a recent study in which 97% of the HBsAg in a 40 μg/ml vaccine preparation was adsorbed on to an aluminum hydroxide adjuvant that had been modified by pretreatment with phosphate using a potassium dihydrogen phosphate at final concentration of 50 mM.¹² In that same study, the percentage of the antigen adsorbed was 100% and 99% with adjuvant pretreated with 0 or 11 mM potassium dihydrogen phosphate, respectively. Importantly, the present study also suggested that incubation at elevated temperatures did not result in changes of adsorbed portion of HBsAg which is another crucial aspect of stability of the vaccine in the new formulations based on 40 mM phosphate (pH 5.2).

It was reported previously⁷ that in a phosphate-free formulation the adsorption of HBsAg onto aluminum hydroxide adjuvant is facilitated mainly by ligand exchange interactions between phospholipids of HBsAg and surface hydroxyls of the adjuvant. Electrostatic interactions were reported to play an insignificant role. It was important to establish in this study whether the surface modification of the adjuvant in the presence of phosphate anion affected the nature of the binding interactions between the antigen and the adjuvant. A potential increase in the importance of electrostatic binding at the expense of the ligand interactions could affect the binding efficiency depending on pH and ionic strength of the formulation with considerable implications for the quality of the vaccine. Electrostatic adsorption of protein antigens onto aluminum salt adjuvants has been described previously to be compromised by high ionic strength of the formulation.¹⁴ However, the present study demonstrated that the surface modification of the adjuvant by phosphate anion (40 mM) did not result in a marked increase of the importance of the electrostatic binding interactions as no considerable effect on the adsorbed portion of the antigen was observed in the presence of sodium chloride up to a 500 mM concentration. The adsorption thus appears to be facilitated by ligand interactions even in the presence of 40 mM phosphate anion, in the same way as reported previously for phosphate-free formulation. This means that ionic species, such as sodium chloride, can be used in the phosphate-based formulations to adjust osmolarity to an optimal level for therapeutic application.

The ionization state of phosphate depends on pH and is determined by its three dissociation constants: $pK_{a1} = 2.2$, $pK_{a2} = 7.2$ and $pK_{a3} = 12.3$ at 25°C.¹⁵ At a pH around 5.2, phosphate exists predominantly in the $H_2PO_4^-$ ionic form and exerts a relatively small buffering capacity. In addition, due to its protonation state at pH 5.2 the buffering capacity of phosphate is stronger on the alkaline side than on the acidic side at such a pH level. Consequently, a formulation of HBsAg based on 40 mM phosphate at pH 5.2 requires additional buffering species. The ability of a chemical species to act as a pH buffer is defined by its dissociation constant (pK_a) which should be within 1 pH unit from the target pH.

However, even species whose pK_a is between 1 and 2 pH units from the target pH can provide some degree of buffering capacity, especially if used at higher concentrations.

The stability of HBsAg adsorbed on aluminum hydroxide adjuvant in the presence of phosphate anion was shown to be further improved in the pH range between 4.6 and 5.2 by the presence of additional ionic excipients. While two of the additional excipients tested, namely malate anion having $pK_{a1} = 3.4$ and $pK_{a2} = 5.1$ at 25°C and succinate anion having $pK_{a1} = 4.2$ and $pK_{a2} = 5.5$ at 25°C,¹⁵ possessed a pK_a in the middle of the pH range studied and therefore provided a strong buffering capacity across the entire pH range, two other excipients, namely histidine having $pK_{a1} = 1.7$, $pK_{a2} = 6.2$ and $pK_{a3} = 9.1$ at 25°C and lactate ion having $pK_a = 3.7$ at 25°C,¹⁵ possessed a pK_a outside the pH range. Consequently, these excipients provided optimal buffering capacity only in part of the pH range studied. However, in all cases, the buffering capacity of the formulation was considerably improved in the presence of the additional excipients over that present in the phosphate-only formulations.

It is noteworthy that the stability of HBsAg appeared to be particularly improved in the presence of excipients that did not comprise an ionizable group with pK_a close (i.e., less than one unit) to the pH of the formulation (i.e., in the presence of lactate anion across the whole pH range studied and in the presence of histidine at pH 4.6 and 5.2). The presence of conventional buffers (i.e., species with pK_a within one unit from the pH of the formulation), namely malate and succinate anions, had only a small or no effect on the stability over that observed in phosphate-only formulations. No further investigations toward elucidating the mechanism of the additional stabilizing effect of histidine and lactate anion were carried out. However, the apparent importance of the pK_a in the stabilizing effect of the selected excipients suggests that exchange of hydrogen cations (H^+) may have a role to play in the degradation of the HBsAg, because excipients with different pK_a have a different ability of exchanging hydrogen cations with ionizable amino acids at the surface of HBsAg. Further investigations using a larger number of additional excipients are needed to elucidate the stabilizing effect.

The optimal formulation for hepatitis B vaccine developed in the course of this study to ensure stability of the antigen during storage comprises phosphate (40 mM) and either histidine (40 mM) or lactate (40 mM) and is adjusted to a pH about 5.2. The HBsAg stability in this formulation is considerably improved over that of the currently marketed hepatitis B vaccine compositions both under heat-stress at 55°C for a few weeks and at more moderate elevated temperatures, 37°C and 45°C, over many months. The animal study performed within this work showed that the stabilized vaccine, after 6 months heat stress, maintained its *in vivo* potency without any change. However, the interpretation of the *in vivo* data may be complicated by the findings on the control vaccine, which only showed a small, but insignificant potency drop. We observed in a subsequent study that longer heat stress was required for the control vaccine to lose potency. Following a 12 month heat stress at 37°C, the control vaccine induced 100-fold lower antibody titer than the stabilized vaccine

and the control vaccine stored at 4°C. This data could not be included in the present paper because the stabilized formulation had an additional excipient for freeze-protection, but will be published separately.

Importantly, all components of the stabilized formulations have a history of safe use in approved drug products, so they may not represent a hurdle in the regulatory approval of the stabilized formulation. Adsorption of antigen onto adjuvant is widely thought to be an important parameter for vaccines containing mineral salt adjuvants. This convention has recently been challenged,¹⁶ and with limited studies, it remains to be determined how antigen dependent is the adsorption requirement. So for a vaccine in which adsorbed antigen has already been demonstrated to elicit a desirable immune response, it is therefore essential to ensure that the adsorption is not compromised by the inactive ingredients in the vaccine formulation. Importantly, it was shown that no significant antigenic activity was measured in the supernatants of the stabilized formulations, indicating that the HBsAg remains strongly bound to the adjuvant in the presence of these excipients. This is very likely due to the strong ligand exchange interactions between the phosphate groups of HBsAg and hydroxyl groups of the aluminum hydroxide adjuvant, even in the presence of 40 mM phosphate. Maintaining the appropriate size distribution of adjuvant particles is another key characteristic essential for the proper functioning of the vaccine, and is therefore one of the main quality parameters of any aluminum salt adjuvant-based vaccine. Importantly, no effect on the size distribution of vaccine particles was observed as a result of vaccine reformulation. The optimized formulation of hepatitis B vaccine reported here thus ensures improved heat stability of the antigen without compromising other key characteristics of the vaccines.

The new formulation of hepatitis B vaccine developed in this work was shown to be stable both at 37°C and at 45°C for at least 6 months using the *in vitro* AUSZYME antigen reactivity test. Although more animal data is needed to confirm the preserved immunogenicity the results shown here indicate that the new formulation of the vaccine can be kept safely at temperatures well above ambient for extended periods of time and has therefore the potential to be used outside the cold chain for part of its shelf life. This may improve immunization coverage and efficiency. The increased stability is very important not only in places where the cold chain is non-existent or insufficient, but also in places where the cold chain is established but temperature deviations may occur during transport or due to other interruptions.

Materials and Methods

Vaccine. Hepatitis B vaccine (Shanvac-B) purchased from Shantha Biotech (Hyderabad, India) was used for this study. Each ml of the Shanvac-B vaccine contains about 20 µg of HBsAg, 0.5 mg of aluminum hydroxide adjuvant, 1.75 mg of disodium hydrogen phosphate, 0.88 mg of potassium dihydrogen phosphate, 7.71 mg of sodium chloride and 25 µg of thimerosal. The vaccine has a pH between 6.9 and 7.1. Solution of HBsAg was obtained from Shantha Biotech.

Materials. Deionized water (analytical reagent grade, Fisher, Loughborough, UK) was used in all experiments. L-histidine, sodium phosphate monobasic, dihydrate, sodium phosphate dibasic, dihydrate and sodium lactate were from Fluka (Gillingham, UK). Sodium chloride, hydrochloric acid and sodium hydroxide were from Fisher. Sodium malate, sodium succinate and TRIS were from Sigma (Poole, UK).

Formulation and stability testing. New vaccine formulations were prepared by centrifuging (14,000 g, 5 min) the original vaccine formulation followed by washing the sediment once with water and resuspending in a background solution containing the new excipients. All background solutions were filtered through a 0.22 µm filter (Millipore, Bedford, MA) prior to their use in new vaccine formulations. The concentration of HBsAg and adjuvant in the new formulations were the same as those in the original Shanvac-B sample (i.e., 20 µg/ml and 0.5 mg/ml respectively). New vaccine formulations were finally transferred into 2 ml glass vials (2-CV, Chromacol, Welwyn Garden City, UK) and sealed with crimp caps. The sealed formulations were placed in an incubator adjusted to one of the following temperatures: 55°C (Incufridge, Revolutionary Science, Lindstorm, MN), 45°C (Lab-Line Instruments, Inc., Model No. 120, Melrose Park, IL), or 37°C (Forma Scientific, Model No. 3326, Marietta, OH) for allotted storage periods. Following the storage, the formulations were tested for remaining antigenic activity and in a particle-sizing assay.

In vitro antigen reactivity assay. The in vitro antigenic reactivity of hepatitis B vaccine was measured using the validated AUSZYME monoclonal diagnostic kit (Abbott Laboratories, Abbott Park, IL). The antigenic reactivity was expressed as a percentage with respect to the value measured of the original refrigerated vaccine. The method required a two-step dilution of each sample in phosphate-buffered saline (1:2,000 v/v) prior to the immunochemical test. Such significant dilution in a buffered solution ensured that the pH of the samples did not affect the result of the test. Exact instructions of the AUSZYME test were followed: The diluted samples were mixed with an antibody-enzyme conjugate reagent and a bead with immobilized monoclonal antibody was added to the mixture and incubated for 75 min at 40°C. The bead was then washed four times using deionized water and dried. Substrate was added to the bead and incubated for 30 min. The reaction was stopped by addition of 1 N H₂SO₄ and absorbance was measured at 492 nm. Each sample was measured in triplicate. In some cases, the adsorbed portion of HBsAg onto the adjuvant was estimated by measuring the antigenic activity both in the whole vaccine and in the supernatant following centrifugation (14,000 g, 5 min). Statistical analyses of the data was performed using Prism 5 software (GraphPad Software Inc., La Jolla, CA). Two-way ANOVA with Bonferroni posttest was used for the comparisons.

It should be noted that the AUSZYME monoclonal diagnostic kit is no longer commercially available. There are, however, alternative commercially available kits to allow measurement of the antigenic reactivity of the hepatitis B vaccine.

In vivo hepatitis B vaccine assay. The in vivo potency was carried out according to the European Pharmacopoeia V5 (p 2.7.15-16) at the National Institute for Biological Standard and Control

(Potters Bar, UK). Briefly, 5 weeks old healthy Balb/C female mice received a single injection of the vaccine intraperitoneally. Each vaccine sample was used at four dilutions (1:24, 1:72, 1:216 and 1:648); 10 mice were used per dilution. The animals were anaesthetized four weeks later and bled. Individual serum was assayed for specific antibodies against HBsAg using the Ausab immunochemical kit. Optical density output of 0.1 was used as the threshold for seroconversion. The percentage of animals showing seroconversion in each group was transformed into the potency of the preparation relative to the control vaccine stored at 4°C, which has a relative potency of 1.

Particle size analysis. A Coulter counter (model Z1, Beckman Coulter, Fullerton, CA) was used to count the number of vaccine particles within several size ranges. Particles were monitored in the following detection ranges: 1.5 to 3, 3 to 6, 6 to 9, 9 to 15, 15 to 20, 20 to 25 and 25 to 30 µm. A 100 µl sample was diluted into 20 ml of ISOTON® II diluent (Beckman Coulter) and assayed for number of particles. Measurements of the diluent without the sample served as the background.

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