Accelerated Stability Study for the Lyophilized Anticancer BCG

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ABSTRACT

Objective: To predict the self-life of the lyophilized BCG during accelerated stability study. Bacillus Calmette and Guerin (BCG) has been commonly known as an anti tuberculosis vaccine since its first introduction in Paris by Calmette and Guerin. Later it was discovered as an important immunotherapeutic agent for treatment of superficial bladder cancer. Only two internationally freeze dried BCG products are approved for bladder cancer treatment worldwide due to several restrictions in WHO guidelines. Other manufactures can only distribute their BCG products in their local markets.

Materials and Methods: BCG was suspended into three stabilizer systems containing 15% w/v trehalose, trehalose-gelatin mixture (in ratio, 30:1 w/w) or lactose. The prepared formulae were lyophilized and the lyophilized formulae were stored at 5◦C, 60% RH for the accelerated study. Scheduled pulling out of samples to test their viabilities was performed according to a stability plan. Shelf-life of each formula was estimated using Q10 method.

Results: Lactose as a stabilizer was found to be superior over trehalose or trehalose-gelatin mixture. Shelf life estimates using Q10 method were about 330 days with Trehalose, 176 days with Trehalose-gelatin and more than two years with lactose compared with 100 days for liquid BCG-T.

Conclusion: Lactose was unique in extending the shelf approximately double the period that was attainable with Trehalose. Meanwhile, Trehalose-gelatin mixture appeared to be the lowest in BCG protection.

Keywords: Bacillus Calmette and Guerin (BCG),lyophilized

INTRODUCTION

Bladder cancer is considered a highly prevalent disease ranked seventh in worldwide cancer incidence affecting more often elderly with median age of 73 at diagnosis[1]. It is the most common malignancy of the urinary tract that accounts for significant morbidity and mortality rates in USA[2]. More than 357,000 new cases are diagnosed worldwide and more than 145,000 deaths are related to urothelial bladder cancer each year[3].

The superiority of BCG in treatment of the rapidly increasing bladder cancer cases was an enough motive for many drug manufacturers to start producing intravesical BCG. Since it is prepared from a live attenuated mycobacterium, BCG must be produced entirely in an aseptic processing environment intended to prevent contamination of the product with other potentially harmful microorganisms[4].

The FDA approved only two BCG products by the United States; "Theracys®" – product of Sanofi Pasteur and "Tice BCG®" – a product of Organon. Other FDA unlicensed products of BCG are being produced to satisfy the local demands of manufacturer countries as with the Indian "BCG ONCO" , the Australian "Immucyst", the Egyptian "BCG-T" and the Tunisian BCG….etc. As mentioned before it took Calmette and Guerin 11 years of culture with 230 consecutive transplants to tame the bacterium and after exporting the strain all over the world, the genetic drifts must have gone further resulting in different therapeutic results and immunogenic properties[5]. This is why urologists do not know whether there is an optimal strain of BCG, but all the known strains are derived from the original strain found by Calmette and Guerin[5].

Based on the knowledge gained form literature about freeze drying cycles and different types of stabilizers for bio-products and for BCG in particular, this work aims to address the effects of freeze drying and changing the type of the stabilizer on BCG stability.

MATERIALS AND METHODS

Materials

The BCG secondary seed lot originated from Japanese laboratories was obtained from the strain store at Vacsera Egypt preserved below -30°C for both production and research purpose, lactose monohydrate (MP Biomedicals, France), trehalose, gelatin, Ziehl Neelsen stain and Karl-Fischer reagent (Sigma-Aldrich, USA), soybean-casein digest, thioglycollate, sauton and Lowenstein Jensen (LJ) media were prepared in Vacsera, Egypt. All other chemicals and solvents were of analytical grade and used without further purification.

Methods

BCG growth, propagation and harvesting

Aseptically, the ampoule of the Japanese BCG seed lot was opened and its contents were gently sprinkled over the surface of the sterilized sauton media and incubated at 37.5°C until a yellowish white corrugated veil of the first generation was developed on the surface of sauton medium (labeled as S1).It was then used for enumerating

propagation of the culture by being transferred, using a special loop, onto the surface of another two sauton media flasks and the same was repeated thrice until the fourth generation of BCG was developed (S4) that had been kept incubated at 37.5°C for 15 days[6]. On the 15th day of incubation, colonies were aseptically harvested and transferred into the glass flask containing stainless steel balls. The bacterial mass was rotated for 3 minutes at 15 rpm using horizontal shaker (Oscillating thermostatically controlled shaker, GallenKamp, England) to be disintegrated.

Preparation of the stabilized formulations

Aqueous solutions containing either 15% w/v trehalose, trehalose-Gelatin mixture (in ratio, 30:1 w/w) or lactose were prepared and sterilized by microfiltration using 0.22 µm millipore filters (GSWP 025 S0, Fisher scientific, USA). The dispersed mass of BCG was added to each cryoprotectant solution to obtain a final concentration of 90 mg/ml of the vaccine[6].

Lyophilization of the prepared formulae

The lyophilization process may be divided into a number of discrete steps: pre-freezing, primary and secondary drying[7-13]. Pre-freezing at -80°c for 48 hours was performed. The used lyophilizer (TFD series, Ilshin Lab Co., Korea) was equipped with a laminar flow hood (LaminAir S-2000 BS II, Holten, Denmark) to ensure the aseptic loading. The vials were taken from the deep freezer (Ultra low temperature freezer, Arktiko Dairer, Denmark) and the stoppers were rapidly loosen by means of a sterile forceps. The vacuum was applied (5 - 9 mtorr) and primary drying was continued for 30 hours, then the temperature was allowed to went up to room temperature by turning the condenser off. When the temperature reached 27-29°C at the same pressure, the secondary drying process started and continued for 6 hours to ensure the maximum drying. The vials were firmly sealed under vacuum by means of a manual down rotation of a piston. Then, vacuum was turned off and the pressure went up till it reached 999 mtorr (atmospheric pressure). The vials were transferred to the refrigerator to be stored at 2-8 C. Samples for the lyophilized samples were reconstituted using sterile WFI for further investigations.

Accelerated stability testing

Twelve samples of each formula were kept at 25°C ± 2°C and 60% RH ± 5% RH in the stability chamber for a period of 210 days. Three samples of each formula were withdrawn on days 30, 90, 135 and 210.

Comparing degradations of the three formulae at accelerated conditions

The obtained viability data for each formula was recorded and the mean values of portion viable “PV” at each sampling time were utilized in the following:

a. Percentages of retained viability
   Percentage of retained viability at the end of the storage period for each formula was estimated to reveal the differences between the three formulae at 25°C and 60% RH.
b. Statistical analysis

Statistical analysis using one way ANOVA of portion viable "PV" at the end of the storage period to test the significance of difference between their abilities to preserve BCG at 25°C and 60% RH.

c. Degradation Profiles
Plot of degradation profiles along the storage period in order to reveal similarities in the trends of degradation at 25°C and 60 % RH.

Shelf life prediction using Q_{10} method

The Q_{10} rule states that the product degradation rate decreases by a constant factor Q_{10} when the storage temperature is decreased by 10^{o} C[14]. Values of Q_{10} are typically set at 2, 3 or 4 but exactly expressed in equation [A][14].

$$\mathbf{Q_{10} = [K_2/K_1]^{[10/T_2-T_1]}} \quad [A]$$

Where K_1 and K_2 are the degradation rate constants at temperatures T_1 and T_2 respectively

The calculated Q_{10} was then employed in equation [B] for shelf life prediction

$$\mathbf{t_{1/2(T2)} = t_{1/2\,(T1)} \,/\, Q_{10}^{\Delta T/10}} \quad [B]$$

Where $t_{1/2}$ (T_2) and $t_{1/2}$ (T_1) are times required for potency to drop to the half at temperatures T_1 and T_2 respectively and $\Delta T = T_2 - T_1$

Calculations were made to predict the shelf lives of the formulae based on the equation of the straight line at accelerated storage conditions.

RESULTS AND DISCUSSION

Accelerated stability data presentation

It was observed that colonies were countable along the 210 days of the test for all the formulae and that viability decreased during the storage period.Data of PV for the three formulae were pooled in table (1) to be compared.

Table 1: The pooled viability data of the accelerated stability study for the three formulae

Pooled accelerated viability data for Trehalose BCG, Trehalose-gelatin BCG and Lactose BCG presented as PV along 210 days			
Time in days	Trehalose-BCG	Trehalose-Gelatin BCG	Lactose-BCG
	0.882	1.072	0.763
30	0.800	0.986	1.091
	0.706	0.804	1.091
	0.329	0.772	0.469
90	0.565	0.643	0.567
	0.235	0.836	0.458
	0.294	0.322	0.818
135	0.353	0.386	0.414
	0.329	0.268	0.240
	0.353	0.107	0.305
210	0.235	0.193	0.316
	0.353	0.129	0.273

Accelerated Stability evaluation

a. Percentages of retained viability.

Percentages of retained viability in accelerated conditions are presented in figure (1).It was observed from figure (1) that the overall retained viability at the end of the test period was the highest with Trehalose BCG 31.4% compared with 29.8% for Lactose BCG and 14.3% for Trehalose-gelatin BCG.

b. Statistical analysis

Data of PV for Trehalose BCG, Trehalose-gelatin BCG and lactose BCG were statistically analyzed to determine the significance of differences in retained viability between formulae at $p \leq 0.05$ in accelerated storage conditions using one way ANOVA using SPSS® 17 software as shown in table (2) followed by Tukey HSD (Honestly Significant Difference) test to determine source of difference as shown in table (3).

It was observed from tables (2 and 3) that 15% Trehalose and 15% Lactose had the same effect of protecting BCG at 25◦C along 210 days whereas 15% Trehalose-0.5%gelatin had significantly lower protective ability in the same conditions. This observation is inconsistent with the hypothesis that polymers, being glass forming additives, exert the highest protection during freeze drying[16]. It might be the ratio of Trehalose/Gelatin that needs to be adjusted by more trials

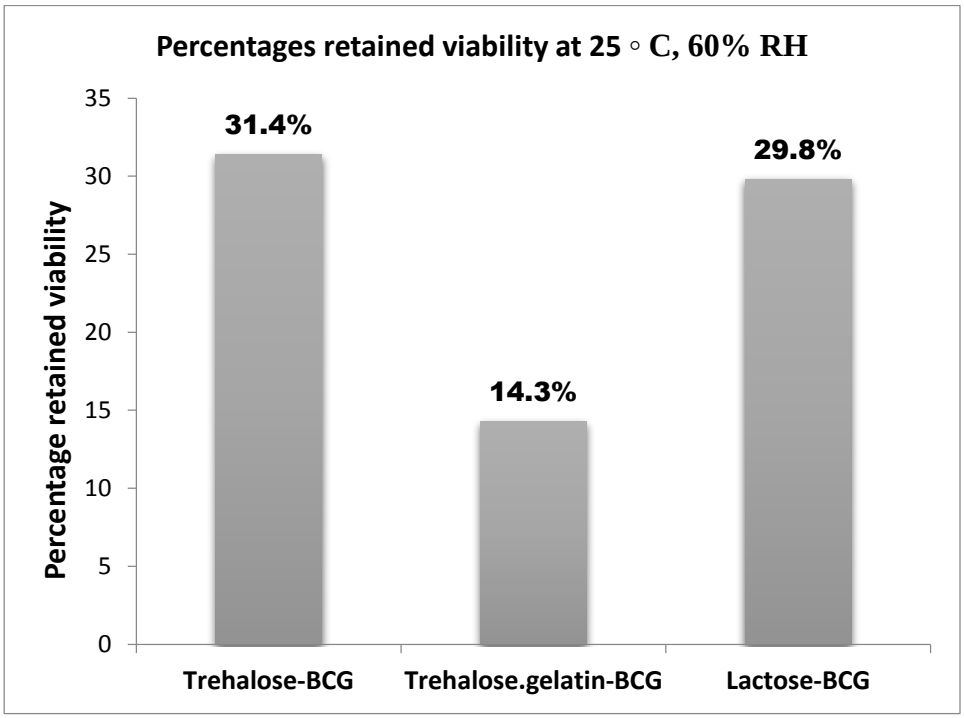

Figure 2. Histogram of percentages retained viability of accelerated stability study forthe three formulae.

Table 2: One way ANOVA test of PV for the three formulae in accelerated storage conditions.

One way ANOVA test of accelerated stability "PV" for Trehalose BCG, Trehalose-gelatin BCG and Lactose BCG along 210 days at $p \leq 0.05$						
		Sum of Squares	df	Mean Square	F	Sig.
30 days	Between Groups	0.06	2	0.03	1.449	0.307
	Within Groups	0.125	6	0.021		
	Total	0.185	8			
90 days	Between Groups	0.218	2	0.109	7.767	0.022
	Within Groups	0.084	6	0.014		
	Total	0.303	8			
135 days	Between Groups	0.055	2	0.027	0.888	0.459
	Within Groups	0.185	6	0.031		
	Total	0.239	8			
210days	Between Groups	0.053	2	0.027	11.224	0.009
	Within Groups	0.014	6	0.002		
	Total	0.068	8			

Table 3: Tukey HSD test of PV for the three formulae in accelerated storage conditions

(1: Trehalose, 2: Trehalose-gelatin, 3: Lactose)

Multiple Comparisons Tukey HSD							
Time in days	Stabilizer system	Stabilizer system	Mean Difference between stabilizers	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
30 days	1	2	-0.158	0.1177	0.425	-0.5191	0.2031
		3	-0.18567	0.1177	0.325	-0.5468	0.1755
	2	1	0.158	0.1177	0.425	-0.2031	0.5191
		3	-0.02767	0.1177	0.97	-0.3888	0.3335
	3	1	0.18567	0.1177	0.325	-0.1755	0.5468
		2	0.02767	0.1177	0.97	-0.3335	0.3888
90 days	1	2	-.37400*	0.09681	0.02	-0.671	-0.077
		3	-0.12167	0.09681	0.467	-0.4187	0.1754
	2	1	.37400*	0.09681	0.02	0.077	0.671
		3	0.25233	0.09681	0.089	-0.0447	0.5494
	3	1	0.12167	0.09681	0.467	-0.1754	0.4187

		2	-0.25233	0.09681	0.089	-0.5494	0.0447
	1	2	0	0.14322	1	-0.4394	0.4394
		3	-0.16533	0.14322	0.519	-0.6048	0.2741
135 days	2	1	0	0.14322	1	-0.4394	0.4394
		3	-0.16533	0.14322	0.519	-0.6048	0.2741
	3	1	0.16533	0.14322	0.519	-0.2741	0.6048
		2	0.16533	0.14322	0.519	-0.2741	0.6048
	1	2	.17067*	0.03982	0.012	0.0485	0.2929
		3	0.01567	0.03982	0.919	-0.1065	0.1379
210 days	2	1	-.17067*	0.03982	0.012	-0.2929	-0.0485
		3	-.15500*	0.03982	0.019	-0.2772	-0.0328
	3	1	-0.01567	0.03982	0.919	-0.1379	0.1065
		2	.15500*	0.03982	0.019	0.0328	0.2772

*. The mean difference is significant at the 0.05 level.

c. *Degradation profiles*

The PV at zero time was set up to 1 and the mean value of portion viable "PV" at each sampling time was calculated from data shown in table (1). Data was recorded as a function of time as presented in figure (2) to show how different formulae behaved over the test period and to determine the closest profiles, if any, for the different used stabilizers[17].

It was clear from figure (2) the close profiles of Trehalose BCG and Lactose BCG along the 210 days unlike a steeper way of degradation of Trehalose-Gelatin BCG in accelerated conditions. It was clear that mixing 0.5% Gelatin with 15% Trehalose diminished its ability in cryoprotection unlike commercially available products such as Warsaw and Moscow BCG stabilized with Saccharose-gelatin mixture[18].

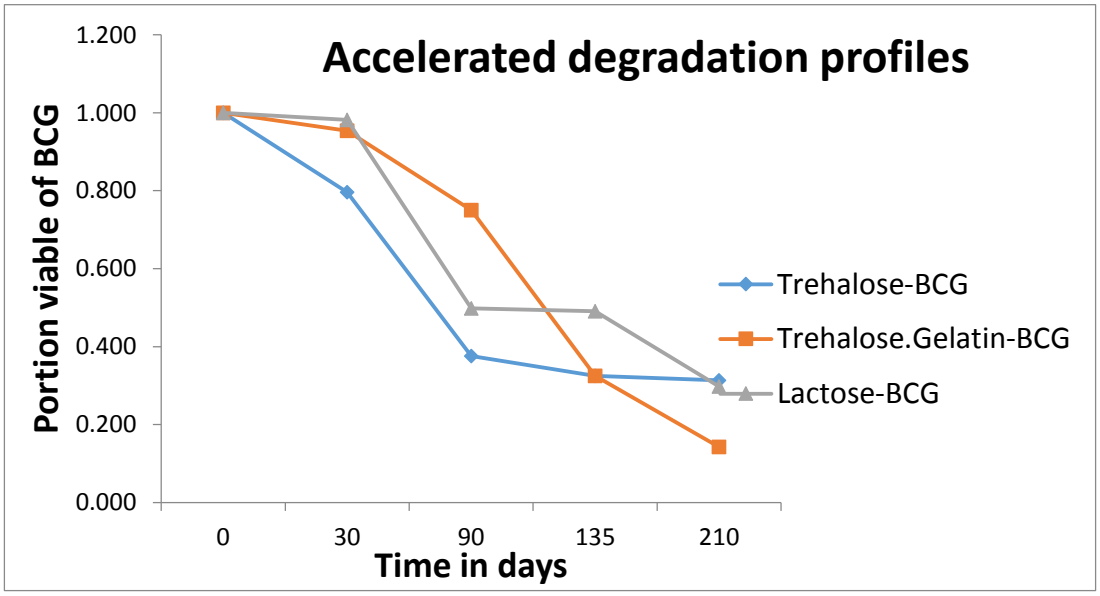

Figure 3: Degradation profile of accelerated stability study for the three formulae.

Shelf life Prediction using Q_{10} method.

The estimated $k_{5°C}$ and $K_{25°C}$ at temperatures 5°C and 25°C respectively were used for substitution in equations [A] and [B]:

<u>Calculations:</u>

1. <u>Trehalose BCG</u>
   a. Accelerated degradation straight line equation
      y = -0.010x + 2.672
   Where y is CFU value at time x and k = -0.010 $CFU.day^{-1}$

   b. The minimum labeled potency for shelf life estimation =
      0.5 x 2.672 = 1.336 CFU

   c. $t_{1/2\ (25)}$ = (2.672 x 0.5) / 0.010 = 133.6 days

   d. Substituting in Q10 formula equation [A] to get the exact Q10 value

      $Q_{10} = [0.010/0.004]^{[10/25-5]} = 1.581$

   e. Substituting in $t_{1/2}$ formula equation [B] to get the predicted shelf life at temperature 5°C

      $t_{1/2(5)} = 133.6 / 1.581^{-20/10} = 333.94$ days

It was proven from the above calculations that the Trehalose BCG formula has a predicted shelf life value of about 334using Q_{10} versus 340 days shelf life using the lower 95% confidence bound in real time data.

2. <u>Trehalose-Gelatin BCG</u>
   a. Accelerated degradation straight line equation
      y = -0.014x + 3.521
   Where y is CFU value at time x and k = -0.014 $CFU.day^{-1}$

   b. The minimum labeled potency for shelf life estimation =
      0.5 x 3.521= 1.761 CFU

   c. $t_{1/2\ (25)}$ = 1.761 / 0.014 = 125.8 days

   d. Substituting in Q10 formula equation [A] to get the exact Q10 value

      $Q_{10} = [0.014/0.010]^{[10/25-5]} = 1.183$

   e. Substituting in $t_{1/2}$ formula equation [B] to get the predicted shelf life at temperature 5°C

$t_{1/2(5)}$ = 125.8 / $1.183^{-20/10}$ = 176 days

It was proved from the above calculations that the Trehalose-Gelatin BCG formula has a predicted shelf life value of about 176 using Q_{10} versus 160 days shelf life using the lower 95% confidence bound in real time data.

3. Lactose BCG

a. Accelerated degradation straight line equation

y = -0.011x + 3.260

Where y is CFU value at time x and k = -0.011 $CFU.day^{-1}$

b. The minimum labeled potency for shelf life estimation =

0.5 x 3.260= 1.630 CFU

c. $t_{1/2\,(25)}$ = 1.630 / 0.011 = 148.18 days

d. Substituting in Q10 formula equation [A] to get the exact Q10 value

$Q_{10} = [0.011/0.002]^{[10/25-5]} = 2.345$

e. Substituting in $t_{1/2}$ formula equation [B] to get the predicted shelf life at temperature 5°C

$t_{1/2(5)}$ = 148.18 / $2.345^{-20/10}$ = 814.85 days

It was proved from the above calculations that the Lactose BCG formula has a predicted shelf life value of about 815 days using Q_{10} method versus an unidentified shelf life beyond 360 days using the lower 95% confidence bound in real time data.

It has to be noted that utilization of accelerated stability data in estimating shelf life using the Q_{10} method was beneficial in this study because the storage temperature of 25° C was not in the neighborhood of the glass transition temperatures *Tg* of the used stabilizers and deformation of the structure wasprevented[19].

CONCLUSION

Upon studying the effects of different stabilizer systems (15% Trehalose, 15% Trehalose-0.5% gelatin mixture and 15% Lactose) on the stability of freeze dried BCG, Lactose as a stabilizer was found to be superior over trehalose or trehalose-gelatin mixture. Shelf life estimates using Q10 method were about 330 days with Trehalose, 176 days with Trehalose-gelatin and more than two years with lactose compared with 100 days for liquid BCG-T.

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