REVIEW

Real time and accelerated stability studies of Tetanus toxoid manufactured in public sector facilities of Pakistan

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Abstract: Tetanus is an acute illness represented by comprehensive increased inflexibility and spastic spasms of skeletal muscles. The poor quality tetanus toxoid vaccine can raise the prevalence of neonatal tetanus. WHO has taken numerous steps to assist national regulatory authorities and vaccine manufacturers to ensure its quality and efficacy. It has formulated international principles for stability evaluation of each vaccine, which are available in the form of recommendations and guidelines. The aim of present study was to ensure the stability of tetanus vaccines produced by National Institute of Health, Islamabad, Pakistan by employing standardized methods to ensure constancy of tetanus toxoid at elevated temperature, if during storage/transportation cold chain may not be maintained in hot weather. A total of three batches filled during full-scale production were tested. All Stability studies determination were performed on final products stored at 2-8ºC and elevated temperatures in conformance with the ICH Guideline of Stability Testing of Biological Products. These studies gave comparison between real time shelf-life stability and accelerated stability studies. The findings indicate long-term thermo stability and prove that this tetanus vaccine can remain efficient under setting of routine use when suggested measures for storage and handling are followed in true spirit.

Keywords: Accelerated stability studies, tetanus toxoid, Pakistan.

INTRODUCTION

Tetanus is an acute, often deadly ailment that is characterized by sweeping augmented stringency and spastic spasms of skeletal muscles (Vandelaer *et al.*, 2003). It remains a major cause of death in establishing countries. In 2008, neonatal tetanus (NT) was probable to have caused >59,000 deaths, accounting for 1% of international infant mortality, first and foremost in establishing nations and is caused by tetanospasmin, a neurotoxin formed by Gram positive bacteria *Clostridium tetani* (Tierney *et al.*, 2012). NT remains a community health problem in many establishing countries including Pakistan, where it is one of the principal causes of neonatal and infant death. In Pakistan, NT accounts for 18-38% and 17-22% of all neonatal and infant deaths respectively (Quddus *et al.*, 2002; Fikree *et al.*, 2002). NT has a high case fatality ratio (CFR) and community-based surveys in developing countries have enunciated CFRs converging 80-90% even with treatment (Whitman *et al.*, 1992). Vaccination is the most steadfast method of forbearance against this disease and has inculcated to lessen mortalities (Parveen *et al.*, 2012). Tetanus vaccine is given to neonates and a pregnant woman, as in mid 1970's it was included in who's Expanded Program on Immunization (Plotkin, 2008). Steadiness of vaccine has a dominant execution in the eminence of immunization programs worldwide (IvanaKnezevic, 2009). The substandard tetanus toxoid vaccine can lead towards the augmentation in the prevalence of neonatal tetanus.

The World Health Organization (WHO) has taken a number of steps to reinforce national regulatory authorities and vaccine manufacturers to safeguard quality of vaccine used in immunization program. It has developed international standards for quality, safety and usefulness of each vaccine which are published in the form of recommendation and guidelines (Joda *et al.*, 2004; WHO, 2004). Stability studies are of two various types such as real time shelf life stability studies and accelerated stability studies. The WHO guidelines have more emphasis on shelf life stability studies which give more accurate measure of stability of vaccine (Ivana-Knezevic, 2009). On the other hand, accelerated stability studies for tetanus vaccine are carried out at ambient temperature (20-25ºC) and at high temperatures (37ºC).

In Pakistan, National Institute of Health, Islamabad is the only public sector organization which is producing tetanus vaccine by formulation, process filling and packaging from imported tetanus concentrates of Tetanus Toxoid. These studies were carried out in NIH Pakistan on three different batches of tetanus toxoid vaccines i.e. 01-06, 02-06 and 03-06. The filling date for these batches was July,

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2006 and expected expiry date at that time for these batches was June, 2009. Vaccines from all three batches were selected randomly and observed for stability under ambient temperature (20-25ºC) and at high temperature (37ºC) for the purpose of accelerated stability studies and under normal storage conditions (2-8ºC) for purpose of real shelf life stability studies. The aim of these studies were to ensure the quality, potency and efficacy of tetanus vaccines produced by National Institute of Health, Islamabad, Pakistan and to ensure stability of tetanus toxoid at elevated temperature if during storage/ transportation cold chain may not be maintained in hot weather. These studies also gave a comparison between real time shelf life stability studies and accelerated stability studies resulting in wider scope of this article.

MATERIALS AND METHOD

Study design

A total of three batches filled during full-scale production were tested. Real time Stability studies determination was performed on final products stored at 2-8ºC and accelerated stability studies was performed on final product stored at 20-25ºC and 37ºC. In conformance with the International Conference on Harmonization (ICH) Guideline of Stability Testing of Biotechnological/ Biological Products, the following parameters were tested: Potency, Sterility test, General safety, and physicochemical parameters i.e Antigen content, Al content, Free Formaldehyde content, Thiomersal content and pH for tetanus toxoid vaccine (BLG/UNDP/77-2 Rev.1). Potency is performed by mouse immunization method according to (BLG/UNDP/77-2 Rev.1).

Requirement

Normal saline, pipettes 5ml, 10ml**,** 100ml Flask, reference vaccine**.** Three dilutions of test as well as reference vaccines are prepared i.e. 1:20, 1:40 and 1:80. The dose of 0.5ml is injected in subcutaneous route on dorsal view of hind limb in a group of 14 mice. After 28 days of immunized mice challenge with tetanus toxin of $50LD_{50}$/dose. Note the death and survival of mice and calculate the potency in term of IU/dose by probit analysis method of WHO (BLG/UNDP/77-2 Rev.1)

Antigenic strength and the purity of tetanus toxoid, prior to its use in the production of vaccine are determined by a flocculation test (to express Lf unit in toxoid). The WHO provides calibrated tetanus toxoid as a primary international standard to help in standardization of assays used to determine the Lf unit of toxoid. It is also used as an antigen for independent in vitro quality control assessments of vaccines and anti-tetanus preparations.

Sterility test

Performed for every sterile component of product as well as on final containers.

Requirement

Pre incubated 35ml Tryptone Soya Broth and Fluid Thioglycollate Medium Tubes**.,** Incubator, one is set at 20 to 25ºC and one is set at 30 to 35ºC**,** Biohazards**,** Disposable Syringes**,** Pre-incubated TSA (Tryptone Soya Agar) plates. Mark the one set of the tubes as media control, one set as Test and one set as Syringe Control. The test is performed by inoculation of sample in Try tone Soya Broth (for aerobic growth) and Fluid Thioglycollate medium (for anaerobic growth). Tryptone broth incubates at 20 to 25ºC and Thioglycollate medium incubate at 30-35ºC for 5-7 days and than transfer in fresh media and incubate on their respective temperature for 14 days.

Interpretation of result

Absence of any type of growth after 14 days incubation of above mentioned media indicates that the samples are sterilized.

General safety

Each final lot of vaccine & sera is tested intra peritoneal injection in mice and g. pig. Two Guinea pigs and five mice are injected for each lot .Inject Single human dose or maximum 1ml in mice and five human doses or maximum 5ml in each guinea pig. The animals are observed for seven days for any abnormality or toxic reaction.

Physicochemical parameters

Antigen content

Antigen content determination is performed according to who guideline on each lot of bulk as well final lot. This test is performed by Ramon titration method to identify the antigen content in the product, the reaction is called flocculation. The antigen content is expressed either Lf/ml or Lf/dose. This is immunological antigen antibody binding test.

Requirement

Reference Antitetanus Serum(equine) 100Lf/ml (ATS)**,** Normal saline(sterile)**,** Water bath set at 50ºC, micropipette (100-100ul)**,** Sodium Citrate. Make the dilution of tetanus toxoid with N. saline then mix with required quantity of ATS, incubate in water bath at 50ºC. Flocculation reaction occurs within one hour Observe the reaction in black background, note the tube which first flocculates. Calculate the Lf/ml by the following Formula: Lf/ml=Amount of antitoxin in ml x Lf/ml of antitoxin/ amount of test sample

Al content determination

Kjeldahl Digestion method: 3ml of well mixed sample is taken in digestion system.

Requirement

Kjedahl digestion system, Digestion flask, -Glass beads, Glass pipettes 1ml,5ml,10ml and 25ml, Erlenmeyer flask (250ml), Magnetic bars, Magnetic stirrer, Sulphuric acid

concentrated 98%, Nitric acid HNO_3 65%, Methyl orange solution 0.1%, Sodium hydroxide NaOH 40%, EDTA 0.02 M, Acetate buffer, pH 4.4, Pyridylazonaphthol solution 0.1%, Copper sulphate ($CuSO_4$) 0.02M

CALCULATION OF RESULT

a=mean consumption of 0.02M $CuSO_4$ for the black, b= mean consumption for the sample, 0.5396= quantity of Al (mg), which is equivalent to 1ml of 0.02M $CuSO_4$-5 H2O or EDTA, V= volume of the sample [(a-b) x 0.5396] /V = mg of Al per ml sample

Free Formaldehyde content determination following requirement:

Acetyl acetone**,** Ammonium acetate**,** Acetic acid (glacial)**,** Formaldehyde 37%**,** Spectrophotometer UV-160**,** Water bath**,** Analytical Balance

CALCULATION

$$\%\text{age of CH2O content} = \frac{\text{Max absorbance of sample}}{\text{Max absorbance of standard}} \times 0.001$$

Criteria for acceptance	Tetanus toxic does not contain more than 0.02% free formaldehyde.
Thiomersal content	

For the determination of thiomersal content, following material are required: Diphenylthio carbazone, Concentrated Nitric acid, Ammonium acetate, Chloroform, Tap water, Separating funnel, Spectrophotometer, Beaker 100ml, Analytical balance, pH meter

CALCULATION

Thiomersal Content = Absorbance of Sample x Conc. of Std (0.01%) Absorbance of std Thiomersal content should be between 0.005- 0.02% pH of the sample of tetanus toxoid final containers should be between 6.0 to 7.0.

RESULTS

Real time stability data congregated from three distinctive clutches of vaccines deliberated under conventional shelf-life circumstances from 2-8ºC for 42 months are illustrated by tables 1, 2 and 3. Consequences for each test have been given after 6 months for individual clutch of vaccines. It has been contemplated that all three clutches clinged desolate amid the cogitation period. The antigenic composition perpetuated its congregation and found to be 20 Lf/ml. Potential or immunogenicity data emulates that applicable vigor of vaccines for the entire period of time was perpetuated and after 42 months found to be (57, 56 and 61) IU/dose for 1, 2 and 3 batches respectively. Accustomed safety and physical manifestation of vaccines are agreeable. Free formaldehyde content, Al content, thiomersal concentration and pH of vaccines dwelled within the ascertained range for the entire period of time are found also illustrated by data. Free formaldehyde content after 42 months come up with 0.00013%, 0.000099% and 0.0044% for 1, 2 and 3 batches respectively. Al content is reported 0.60 for batch 1 and 0.56 for both batches 2 and 3. pH of batch 1 vaccines exhibited no change in value and remains 6.49 after 42 months, pH of batch 2 and batch 3 vaccines exhibited a very small drop in value from 6.68-6.65 and 6.55-6.45 respectively but still fall well within limits i-e 6-7. It can be consummated that vaccines perpetuated their efficacy,

Table 1: Real time stability data of tetanus toxoid vaccine formulated in 2007

Test Item	Specification	Initial (July 2007)	After 6 month (Jan.2008)	After 12 months (Sep 2008)	After 18 months (March 2009)	After 24 months (Sep 2009)	After 30 months (March 2010)	After 36 Months (July 2010)	After 42 months (Jan 2011)
Sterility	No growth In TSB & FTM	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Antigen content	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml
Potency	Not less 40IU/dose	56 IU/dose	68IU/dose	68 IU/dose	56IU/ml	65IU/dose	60IU/ml	62IU/dose	57IU/dose
G. Safety	Animal remains alive	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Free Formaldehyde content	Not more than 0.02%	0.00013%	0.00013%	0.00011%	0.00013%	0.00012%	0.00013%	0.00011%	0.00013%
Al content	Not more than 1.25mg/dose	0.62	0.62	0.60	0.61	0.60	0.59	0.62	0.60
Thiomersal	0.005 to 0.02%	0.0089%	0.0090%	0.0091%	0.0089%	0.0088%	0.0087%	0.0090%	0.0089%
pH	6.0 to 7.0	6.49	6.45	6.50	6.40	6.30	6.44	6.50	6.49
Physical appearance	Gel content remains uniform through out the batch	Satisfactory	Satis-factory	Satis-factory	Satis-factory	Satis-factory	Satis-factory	Satis-factory	Satis-factory

Table 2: Real time stability data of tetanus toxoid vaccine formulated in 2007

Test Item	Specification	Initial (July 2007)	After 6 month (Jan.2008)	After 12 months (Sep 2008)	After 18 months (March 2009)	After 24 months (Sep 2009)	After 30 months (March 2010)	After 36 months (July 2010)	After 42 months (Jan 2011)
Sterility	No growth In TSB &FTM	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Antigen content	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml
Potency	Not less 40IU/dose	62IU/dose	63 IU/dose	51 IU/dose	67IU/dose	65 IU/dose	69IU/dose	66 IU/dose	56 IU/dose
G.safety	Animal remains alive	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Free Formal dehyde content	Not more than 0.02%	0.000098%	0.000099%	0.000095%	0.000099%	0.000094%	0.000099%	0.000096%	0.000099%
Al content	Not more than 1.25mg/dose	0.59	0.60	0.62	0.58	0.62	0.58	0.59	0.56
Thiomersal	0.005 to 0.02%								
pH	6.0 to 7.0	6.68	6.56	6.66	6.57	6.70	6.66	6.46	6.65
Physical appearance	Gel content remains uniform through out the batch	Satisfactory	Satisfactory	Satisfactory	Satisfactory	Satisfactory	Satisfactory	Satisfactory	Satisfactory

Table 3: Real time stability data of tetanus toxoid vaccine formulated in 2007

Test Item	Specification	Initial (July 2007)	After 6 month (Jan.2008)	After 12 months (Sep. 2008)	After 18 months (March2009)	After 24 months (Sep.2009)	After 30 months (March 2010)	After 36 months (July 2010)	After 42 months (Jan 2011)
Sterility	No growth In TSB & FTM	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Antigen content	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml
Potency	Not less 40IU/dose	62IU/dose	63IU/dose	63IU/dose	63IU/dose	68IU/dose	60Iu/dose	68IU/dose	61IU/dose
G.safety	Animal remains alive	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Free Formalde hyde content	Not more than 0.02%	0.0043%	0.0040	0.0040	0.0043	0.0042	0.0044	0.0041	0.0044
Al content	Not more than 1.25mg/dose	0.54	0.58	0.55	0.59	0.54	0.52	0.55	0.56
Thiomersal	0.005 to 0.02%	0.0091	0.0089	0.0092	0.0089	0.0092	0.0091	0.0092	0.0093
pH	6.0 to 7.0	6.55	6.45	6.25	6.55	6.22	6.35	6.55	6.45
Physical appearance	Gel content remains uniform through out the batch	Satisfactory	Satisfactory	Satisfactory	Satisfactory	Satisfactory	Satisfactory	Satisfactory	Satisfactory

quality and potency throughout the shelf life period.

Accelerated stability study data elicited from the tests on vaccines maintained under storage conditions from 20-25ºC for twelve months are supported by tables 4, 5 and 6. pH of these vaccines exhibited a very small drop in value from 6.49 to 6.50, but still fall well within limits. There is no dilemma with the sterility and physical emergence of these vaccines, both are cogent. Antigenic content clings at the level of 20 Lf/ml throughout twelve months in all three batches. It also shows that vaccines remained effective and safe for use. During this twelve months accelerated study, all these parameters remained fine within limits i-e for 4, 5 and 6 batches; free formaldehyde content, Al content and thiomersal concentration come up with 0.00011%, 0.60 and 0.0091% respectively.

Tables 7, 8 and 9 depicts accelerated stability study data aggregated from examining vaccines which were hold back under high temperature of 37ºC for six months. pH of these vaccines exhibited a very small drop in value

Table 4: Accelerated stability data of tetanus toxoid vaccine formulated in 2007

Test Item	Specification	Initial	After 3 months	After 6 month	After 9 months	After 12 months
Sterility	No growth In TSB & FTM	Pass	Pass	Pass	Pass	Pass
Antigen content	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml
Potency	Not less 40IU/dose	56 IU/dose	62 IU/dose	60IU/dose	56IU/dose	57 IU/dose
G.safety	Animal remains alive	Pass	Pass	Pass	Pass	Pass
Free Formal dehyde content	Not more than 0.02%	0.00013%	0.00011%	0.00013%	0.00013%	0.00011%
Al content	Not more than 1.25mg/dose	0.62	0.60	0.62	0.62	0.60
Thiomersal	0.005 to 0.02%	0.0089%		0.0090%		0.0091%
pH	6.0 to 7.0	6.49	6.50	6.45	6.48	6.50
Physical appearance	Gel content remains uniform through out the batch	Satisfactory	Satisfactory	Satisfactory	Satisfactory	Satisfactory

Table 5: Accelerated stability data of tetanus toxoid vaccine formulated in 2007

Test Item	Specification	Initial	After 3months	After 6 month	After 9 months	After 12 months
Sterility	No growthIn TSB & FTM	Pass	Pass	Pass	Pass	Pass
Antigen content	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml
Potency	Not less 40IU/dose	62 IU/dose	58 IU/dose	61 IU/dose	59IU/dose	60IU/dose
G. Safety	Animal remains alive	Pass	Pass	Pass	Pass	Pass
Free Formal dehyde content	Not more than 0.02%	0.00013%	0.00011%	0.00013%	0.00011%	0.00011%
Al content	Not more than .25mg/dose	0.62	0.60	0.62	0.60	0.60
Thiomersal	0.005 to 0.02%	0.0089%	0.0091%	0.0090%	0.0089%	0.0091%
pH	6.0 to 7.0	6.49	6.50	6.45	6.45	6.50
Physical appearance	Gel content remains uniform through out the batch	Satisfactory	Satisfactory	Satisfactory	Satisfactory	Satisfactory

Table 6: Accelerated stability data of tetanus toxoid vaccine formulated in 2007

Test Item	Specification	Initial	After 3 months	After 6 month	After 9 months	After 12 months
Sterility	No growth In TSB & FTM	Pass	Pass	Pass	Pass	Pass
Antigen content	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml
Potency	Not less 40IU/dose	62 IU/dose	56.5IU/dose	60 IU/dose	60IU/dose	60 IU/dose
G.safety	Animal remains alive	Pass	Pass	Pass	Pass	Pass
Free Formal dehyde content	Not more than 0.02%	0.00013%	0.00013%	0.00013%	0.00011%	0.00011%
Al content	Not more than 1.25mg/dose	0.62	0.62	0.62	0.62	0.60
Thiomersal	0.005 to 0.02%	0.0089%	0.0090%	0.0090%	0.0089%	0.0091%
pH	6.0 to 7.0	6.49	6.45	6.45	6.49	6.50
Physical appearance	Gel content remains uniform through out the batch	Satisfactory	Satisfactory	Satisfactory	Satisfactory	Satisfactory

from 6.49 to 6.45, but still fall well within limits. Sterility and physical emergence both remained at cogent level. Potency and general safety halted same except, some aberrations in potency of batch no. TT-AA-07, still all assessments were within the limits. Al content is detected 0.62, free formaldehyde concentration is 0.00013%, antigenic content is 20 Lf/ml and thiomersal concentration is 0.0090% for all 7, 8 and 9 batches. All parameters emerge normal and adequate in all three tables. Thus these 6 month interval studies found to be highly stable than the above two kind of studies.

DISCUSSION

Vaccines and sera's are not administered with dubious potency, but rather within a specific range that is established through irrefutable clinical studies. All molecular entities, including all vaccines, will degrade over time, resulting in the case of vaccines in a loss of potency (Jodar *et al.*, 2004; Egan and Schofield, 2009). World Health Organization (WHO) recommends adequate stability studies for the production and control of vaccines as an essential part of vaccine development (Ivana-Knezevic, 2003). The data on retained vaccine potency at

Table 7: Accelerated stability data of tetanus toxoid vaccine formulated in 2007

Test Item	Specification	Initial	After 3 months	After 6 month
Sterility	No growth In TSB & FTM	Pass	Pass	Pass
Antigen content	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml
Potency	Not less 40IU/dose	56 IU/dose	57IU/dose	57IU/dose
G. Safety	Animal remains alive	Pass	Pass	Pass
Free Formaldehyde content	Not more than 0.02%	0.00013%	0.00013%	0.00013%
Al content	Not more than 1.25mg/dose	0.62	0.62	0.62
Thiomersal	0.005 to 0.02%	0.0089%	0.0089%	0.0090%
pH	6.0 to 7.0	6.49	6.49	6.45
Physical appearance	Gel content remains uniform through out the batch	Satisfactory	Satisfactory	Satisfactory

Table 8: Accelerated stability data of tetanus toxoid vaccine formulated in 2007

Test Item	Specification	Initial	After 3 months	After 6 month
Sterility	No growth in TSB & FTM	Pass	Pass	Pass
Antigen content	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml
Potency	Not less 40IU/dose	62 IU/dose	61 IU/dose	60 IU/dose
G. Safety	Animal remains alive	Pass	Pass	Pass
Free Formaldehyde content	Not more than 0.02%	0.00013%	0.00013%	0.00013%
Al content	Not more than 1.25mg/dose	0.62	0.62	0.62
Thiomersal	0.005 to 0.02%	0.0089%	0.0089%	0.0090%
pH	6.0 to 7.0	6.49	6.49	6.45
Physical appearance	Gel content remains uniform through out the batch	Satisfactory	Satisfactory	Satisfactory

Table 9: Accelerated stability data of tetanus toxoid vaccine formulated in 2007

Test Item	Specification	Initial	After 3 months	After 6 month
Sterility	No growth In TSB & FTM	Pass	Pass	Pass
Antigen content	20Lf/ml	20Lf/ml	20Lf/ml	20Lf/ml
Potency	Not less 40IU/dose	62 IU/dose	61 IU/dose	61IU/dose
G. Safety	Animal remains alive	Pass	Pass	Pass
Free Formaldehyde content	Not more than 0.02%	0.00013%	0.00012%	0.00013%
Al content	Not more than 1.25mg/dose	0.62	0.64	0.62
Thiomersal	0.005 to 0.02%	0.0089%	0.0089%	0.0090%
pH	6.0 to 7.0	6.49	6.49	6.45
Physical appearance	Gel content remains uniform through out the batch	Satisfactory	Satisfactory	Satisfactory

temperatures other than the recommended refrigerated storage temperature are clinically useful because cold chain conditions cannot always be maintained. Furthermore, studies under stress conditions may be useful in determining whether short term accidental exposure to undesired conditions, such as during transport, can compromise product quality. Post marketing stability studies of vaccines that tend to be close to their clinical specification at the end of the expiration dating period may require enhanced annual monitoring. In addition, an early assessment of product stability prior to completion of each individual study is desired. However, predictive measures of individual lots may produce early indication of failure. In many cases, these prove to be false alarms. For such products, continued product quality after marketing should, therefore, depend less on evaluating individual observations or individual lot projections, and more on assuring that the underlying stability profile of the product as a whole has not changed (Fairweather *et al.*, 2003).

Our data collected from three different batches of vaccines deliberated under orthodox shelf-life conditions as mentioned above in results, shown to have acquainted assurance and physical revelation of vaccines. Moreover the free formaldehyde content, Al content, thiomersal concentration and pH of vaccines lodged amid the determined range for the integrated period of time and conservation of efficacy, quality and strength of vaccines throughout the shelf-life period were found to conform the WHO's limits. Stability studies also play a critical role in assuring product quality at all points in the vaccine life cycle. At and after licensure, stability studies on quality attributes (including potency) provide a critical link

between marketed and clinically evaluated vaccine product, addressing important regulatory concerns by assuring that product quality is maintained throughout the dating period. During development, stability studies are done to assure product quality and to obtain the data needed to support licensure. Stability studies may also be performed after licensure to assure that product continues to perform as it did pre-licensure, as well as to evaluate the effect on product quality of deliberately introduced manufacturing changes. At each phase in the product life cycle, it is important to consider the goals of stability evaluation and to perform appropriate statistical analyses in order to assure and reach appropriate conclusions about product quality (Krause, 2009).

Our studies showed no degradation or loss of potency during elevated temperature and in real life studies which shows that stability of the formulation. It is suggested that an understanding of the principles of degradation, as well as the statistical tools for measuring product stability, is essential for the management of product quality. Key to this is management of vaccine potency. The loss of potency of the vaccine can leads towards the prevalence of neonatal tetanus. The determinants of the incidence of NT relate to the cultural diversity of hygienic childbirth practices and cord care Bennett *et al.,* 1996; Quddus *et al.,* 2002 as well. The lack of skilled attendance with delivery, parent's illiteracy, lack of antenatal care, including low level of immunization against tetanus, seasonality, geographical location and climate prevalence of spores of C. tetani and rural agricultural settled populations (Daud *et al.,* 1981; Harfouche, 1982; Kessel, 1984) are some of the factors responsible for the disease.

Vaccine shelf-life is best managed through determination of a minimum potency release requirement, which helps assure adequate potency throughout expiry. Use of statistical tools such a least squares regression analysis should be employed to model potency decay. The use of such tools provides incentive to properly design vaccine stability studies, while holding stability measurements to specification presents a disincentive for collecting valuable data. The laws of kinetics such as Arrhenius behavior help practitioners design effective accelerated stability programs, which can be utilized to manage stability after a process change. Design of stability studies should be carefully considered, with an eye to minimizing the variability of the stability parameter. In the case of measuring the degradation rate, testing at the beginning and the end of the study improves the precision of this estimate. Additional design considerations such as bracketing and matrixing improve the efficiency of stability evaluation of vaccines (Egan and Schofield, 2009). In our study, the testing of the vaccines has been done every six months till it attains expiry and has also extended its testing for another six months. The results were found to be very satisfactory. It shows that annual stability program also helps assure continued quality of product throughout the dating period, while comparability studies are performed after a process or facilities change in order to demonstrate that the change has not impacted the stability characteristics of the product. Careful attention to the design and analysis of post licensure studies helps mitigate the risk of missing a meaningful shift in the degradation rate of a vaccine, as well as the possibility of incorrectly earmarking a stability shift when the product remains acceptable (Schofield, 2009). Evaluation of stability is an essential part of the assessment of the vaccine quality. Indeed, the stability studies are aimed at verifying that the vaccines maintain their original quality criteria throughout their shelf lives (Socarras and Magari, 2009). It has also been reported that if certain vaccines are stored unopened at the recommended temperature (20°C), the freeze-dried material is highly stable with a predicted degradation rate of 0.032% loss of activity per year (Sesardic, 2010).

It is worth mentioning that excursions from storage condition requirements may affect product performance and stability. The effects of temperature excursion on stability depend on the amount of time that a product is subjected to these conditions, temperature level and activation energy. Both time at elevated temperature and the temperature level can be directly measured, while activation energy needs to be estimated from the accelerated stability tests. Coulter Clenz reagent degradation information is used to demonstrate the effects of temperature excursions. The stability of the product is affected by any excursion, but Coulter Clenz will not lose all of its stability for excursion of up to 30 days at 35ºC and 20 days at 40ºC Temperature excursion for up to 20 days at 40ºC will reduce the stability of a product that has activation energy in the range of 26-30 kcalmol (-1) approximately by 5-7 months. Products with lower activation energy will have a significantly lower reduction in stability. The effects of excursions on shelf life performance are less severe when lower level of risk is implemented to establish the claimed shelf life. The proposed model can effectively predict temperature excursion if used within the scope of a product performance and its characteristics (Socarras and Magari, 2009).

The findings of our studies carried out at National Institute of Health, Islamabad, Pakistan are quite comparable to those studies done by GSK, USA (Schofield, 2009) but were unique in nature as they are conducted for the first time in Pakistan. The Impact of these studies will be in the promotion of locally manufactured vaccine usage and helps in saving millions of dollars required for the purchase of imported vaccines and will lessen the financial burden on national exchequer. These studies will also redress conspiracies regarding quality, efficacy and potency of tetanus

vaccines produced by National Institute of Health, Islamabad Pakistan. The scope of these studies also includes a positive impact on reducing neonatal deaths because of this preventable disease and promotion of Expanded Program of Immunization program in Pakistan. The present findings also indicate long-term thermo stability and provide declaration that this tetanus vaccine can remain efficient under setting of routine use when suggested measures for storage and handling are followed in true letter and spirit.

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