



RESEARCH ARTICLE

BIOCHEMISTRY

**COMPARATIVE ANALYSIS OF MAGNESIUM CHLORIDE IN RECENTLY DEVELOPED LIQUID STATE RABIES VACCINE**

**JAGANNATHAN S<sup>1\*</sup>, MANI K.R<sup>2</sup>., VIJAYAKUMAR R<sup>3</sup>., RAHUL GANDHI P<sup>4</sup> AND VENKATARAMANA K.N<sup>5</sup>.**

<sup>1</sup>Assistant Research Officer, Tissue Culture anti Rabies Vaccine Production laboratory, Pasteur Institute of India, Coonoor, Tamilnadu, India. PIN 643 103.

<sup>2</sup>Deputy Director, Pasteur Institute of India, Coonoor, Tamilnadu, India. PIN 643 103.

<sup>3</sup>Department of Biochemistry, Karpagam Arts and Science College, Coimbatore, Tamilnadu, India. PIN 641 021.

<sup>4</sup>Department of Biotechnology, Jamal Mohammed College, Trichirapalli, Tamilnadu, India. PIN 621 021.

<sup>5</sup>Assistant Director, Pasteur Institute of India, Coonoor, Tamilnadu, India. PIN 643 103.



**JAGANNATHAN S**

Assistant Research Officer, Tissue Culture anti Rabies Vaccine Production laboratory, Pasteur Institute of India, Coonoor, Tamilnadu, India. PIN 643 103.

\*Corresponding author

**ABSTRACT**

Rabies is normally a rapidly fatal neurological disease and to date therapeutic efforts in humans have proved futile except in rare cases in which rabies vaccine was administered prior to the onset of clinical disease. Safe and potent vaccines produced by cell culture techniques are available in developed countries. One of the most elements in the effective control of human rabies is the use of efficacious vaccines. The stabilizers play an important role in the efficacy of vaccines. Our present study highlights the analysis of Magnesium Chloride as a stabilizer in the newly developed vero cell derived rabies vaccine.



## KEYWORDS

Rabies, MgCl<sub>2</sub>, Titan yellow, Erichrome black- T, Human albumin.

## INTRODUCTION

Rabies is lethal encephalitis caused by a lyssa virus which is transmitted from animals to humans via bite wounds, or licking of mucous membranes (**Hemachudha et al., 2002**). The number of annual human deaths is unknown but is thought to be well over 50 000. Nearly 50% of these deaths are in children. They are less able to defend themselves against biting animals than adults, and are more likely to be bitten on high-risk body parts such as the face, head, and hands (**WHO, 2005**). Rabies is responsible for more deaths than polio, yellow fever, Japanese encephalitis, SARS, or meningococcal meningitis. India alone had an estimated 30 000 annual rabies deaths during the past decades (**Wilde et al., 2005**). Asian countries reported as high as 20,000 human rabies deaths annually. Rabies in India occurs mainly in the urban form (**Sudarshan et al., 2007**). Vaccines are widely used as highly cost-effective tools for improving health and have for the last decades had a major impact on public health. Vaccine stabilization strategies must include efforts to screen and identify appropriate stabilizers to prevent the vaccine inactivation (loss of viability) under environmental stress; such as elevated temperatures. Stabilizers are chemical compounds added to the vaccine and are used in conjunction with either lower temperature storage or lyophilization methods (**McAleer et.al., 1979**). Various stabilizers are added to vaccines. For freeze dried (lyophilized) preparations of vaccines, it is additionally necessary to add materials that provide a bulk matrix for the vaccine. The amount of an immunogen that is contained in a vaccine can be extremely small, on the order of

tens of micrograms or less. If sufficient amounts of various materials were not added to the vaccine prior to lyophilization, the vaccine would not be readily observable and would undoubtedly adhere to the wall of the vaccine vial.

But during the freeze drying process there is a losses of considerable antigenicity (immunogenic, potency). The residual moisture content is a mandatory requirement to specify the water content of vaccines distributed for immunization. The high moisture contents from the lyophilizer can result in poor stability, since sufficient free water may remain in the samples to permit conformational changes in a bio molecule (**Greiff D 1991**) and also it should be in the prescribed level of below 3% (**IP 2007**). Residual moisture reduces the potency and stability of the viral proteins. So, during the freeze drying, the viral proteins are supplemented with stabilizers and additives to maintain the stability. Human albumin and Maltose are very efficient stabilizer and additives for rabies vaccine. As per the IP 2007 the Single Human Dose (SHD) consists of the purified rabies antigen, Stabilizer, Additives and adjuvants are 2.5 IU, 5%, 1% and less than 2.5 mg respectively.

The basic problem in dealing with preservation of viral vaccine is the loss in virus titer either during lyophilization or due to improper cold chain conditions. Due to the worldwide distribution of vaccines and the diversity of ambient temperature, there has been a need to stabilize these preparations for transportation and use. Several stabilization methods have been used in the past (**Jagannathan et.al., 2010**).



All the viral harvest of PTARV-2 (Pasteur Tissue Culture Rabies Vaccine-2) are pooled and concentrated by Pellicon cassette system (Millipore) (**Jagannathan et al, 2008**). Concentrated viral harvests are inactivated with  $\beta$ -Propiolactone. The entire inactivated antigen are purified and formulated with human albumin and magnesium chloride. Prior to release the vaccines are inspected of their standard by the guidelines of Indian Pharmacopeia.

Magnesium chloride is used as the stabilizer as well as adjuvants in oral polio vaccine for human use (**WHO TRS 1987**). The Magnesium chloride is boosting the immune response when combined with polio vaccine (**Shoekey et al. 1988**). Oral polio vaccine stabilized with magnesium chloride is quite a stable vaccine and maintenance of a proper cold chain is recommended for the delivery of a potent vaccine in countries with high ambient temperature (**Mirchamsy et.al., 1978**).

Additives are used to stabilize the vaccines from adverse conditions such as freeze drying and heat. In addition, additives are added to vaccines to prevent immunogens from adhering to the side of the vial. Human Serum Albumin is the most abundant protein in plasma, is a major antioxidant, transport, and depot protein. This globular 66 KDa protein contains 585 amino acids including 18

tyrosines, 6 methionines, 1 tryptophan, 17 disulfide bridges, and only 1 free cysteine (Cys34). Many commercial HSA preparations come as a sterile aqueous solution prepared by a cold alcohol fractionation method from pooled human plasma obtained from venous blood. The human serum albumin product is typically at 20% (20 g/100 ml). It is used predominantly in viral vaccines (**Plotkin 2006**). And it is also suggested by WHO for use as a additives (**WHO 2007**).

Monolayers of VERO cell line is inoculated and infected with strains of Pasteur virus (PV11). The virus and viral protein are harvested from infected cultures from the third day after inoculation, five times at the interval of 72hrs each, the virus titres was above the WHO prescribed level ( $10^{5.2}$ ). Subsequently single viral harvest is pooled and concentrated by tangential flow filtration (TFF) using Pellicon system and inactivated by beta propiolactone. Subsequently it is further purified by chromatographic technique. Concentrated, inactivated, purified rabies viral proteins are formulated with Magnesium Chloride and Human Albumin (Table 1). All the reagents were of analytical grade (E MERCK India, Ltd) and only deionized MilliQ water was used for samples and standard preparation (**Jagannathan et.al., 2010**).

**Table 1**  
**Constituents of Liquid rabies vaccine (TCALRV- B)**

Vaccine contents	Rabies antigen	1M Magnesium Chloride	Human Albumin	IU/100ml	IU/SHD
Volume (in ml)	85	10	5	603.5	6.03



## MATERIALS

1. Newly formulated TCALRV-B (Tissue Culture Anti Liquid Rabies Vaccine) obtained from anti rabies vaccine production section of The Pasteur Institute of India along with its production details.
2. 1M Magnesium Chloride
3. 20% Human Albumin Human Normal Albumin. TP, *ALBUREL*<sup>TM</sup> ( For IV use), Reliance Life Sciences, Mumbai.
4. 10% Trichloro acetic acid.
5. Gumghatti solution: 1g of gum ghatti is tied loosely in muslin cloth and left suspended in 1litre of distilled water overnight. The soluble part of the gum leaches out into the water. The residue is discarded and the solution preserved with a few drops of chloroform.
6. 0.05% Titan yellow solution
7. Sodium hydroxide solution 4N
8. Erichrome Black T (0.1% in methanol)

## METHODS

### Analysis of magnesium by titan yellow method:

The proteins in the vaccine were precipitated using tri-chloro acetic acid. Titan yellow solution is added to the supernatant with the presence of gumghatti which maintains the dispersion and 4N of sodium hydroxide is added. The developed colour was read at 520nm spectrometrically (**Muller et.al., 1994**).

100µl of vaccine sample was diluted to 500µl with sterile distilled water and protein gets precipitated by adding 200µl of 5% trichloro acetic acid. Then centrifugation is carried out at 2500rpm for 15minutes and 250µl of supernatant was collected. Then 100µl of gumghatti, 100µl of titan yellow and 100µl of 4N NaOH were added. Then the colour development was read at 520nm spectrophotometrically. Simultaneously blank

was set with 250µl of sterile distilled water and followed the same procedure.

### Analysis of magnesium by Erichrome black-T method:

200µl of vaccine was taken and the volume was made up to 1ml with sterile distilled water. After that 1ml of 10% TCA was added and mixed well using cyclomixer. Then it was allowed to stand for 15 minutes and it was filtered using whattman filter paper. 1ml of that filtrate was taken and 300µl of 8% ammonia and 500µl of ammonia buffer were added and mixed. Then 200ml of dye solution (0.1% dye in methanol) was added. The colour development was read at 520nm spectrometrically (**Durham AC et.al., 1983**).

## RESULTS AND DISCUSSION

MgCl<sub>2</sub> is boosting the immune response when combination with the viral protein (**Rajput et.al, 2010**). We need further research work regarding the combination of MgCl<sub>2</sub> with rabies viral protein in the presence of human serum albumin. In the presence of human albumin, the association between MgCl<sub>2</sub> and rabies viral protein is found to elevate the immune in the target animals.

In the presence of Human Serum Albumin the binding strength of rabies viral protein and MgCl<sub>2</sub>, shows higher degree of immune response. In general for the production of immunoglobulin, the immunobiologicals should formulate with commercially available adjuvants for increasing the strength of immune response. But when the same immunobiologicals are formulated with MgCl<sub>2</sub>, there is no need of commercial adjuvants. In the result more encouraging for the utilization of MgCl<sub>2</sub> with rabies viral protein in a liquid state which needs further studies in the case of plantibodies, oral rabies, veg vaccines or edible plant mediated



biopharmaceuticals (**Jagannathan et.al., 2008**)

The proteins present in the liquid rabies vaccine are not an influential factor for the efficacy of vaccines. Only its antigenicity (immunogenicity) of inactivated, purified rabies viral proteins plays the main role in the efficacy (**Vijayakumar 2010**). All the formulated

vaccines potencies are above the WHO recommended level (**Table I**). The liquid vaccine samples are further assayed with slight modification of various techniques involved for the determination of magnesium. All the experiments are done in duplicates and their means are calculated and tabulated (**Table 2 & 3**).

**Table 2**  
**Magnesium analysis in vaccine sample.**

Vaccine name	Titan yellow method			Erichrome Black –T method		
	EXP 1 (%)	EXP 2 (%)	Average (%)	EXP 1 (%)	EXP 2 (%)	Average (%)
TCALRV- B	18.8	21.3	20.0	18.6	19.3	18.9

**Table 3**  
**Amount of magnesium in liquid rabies vaccine during and after formulation.**

Vaccine name	During formulation	After formulation	
		Titan yellow	Erichrome Black- T
TCALRV- B	20.3%	20.0%	18.9%

In the titan yellow method, the amount of magnesium was estimated as 20 grams which has the deviation of 0.3%. In the Erichrome black- T, method the amount of magnesium in the vaccine sample showed 18.9grams and the deviation was found to be as 1.4% (**Table 4**). The titan yellow method showed the satisfactory result with the production amount of magnesium compound which was added in the viral protein. This method is easy to analyse and can complete in

short time. The colour development is stable for three hours and the reagents prepared for the analysis also can be stored for long use. But in case of the Erichrome black-T method the possibility chance of error are more. It is time consuming and filtration process is required to separate the proteins in the vaccine. The main disadvantage of the method is, the colour development which is stable up to 5 minutes only.



**Table 4**  
**Deviation of magnesium in formulated liquid rabies vaccine.**

Name of vaccine	Titan yellow method	Erichrome method	Black-T method
TCALRV-B	20.3-20.0= 0.30*	20.3-18.9= 1.4*	

\*A-B=C

Where A = formulation percentage

B= Average test values

C= Difference between formulation percentage and average test values.

From these experiments, although both methods show satisfactory results, titan yellow method is simple, easy and accuracy is also good comparing to the Erichrome black- T method.

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