

Analysis of Carbohydrates in Newly Developed Liquid State Rabies Vaccine

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Abstract

Rabies is an under-reported, neglected deadly disease estimated to cause more than 50,000 human deaths annually, most of which occur in the poorest regions of the world. 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 the vaccines. Our present study emphasizes on the analysis of stabilizers, trehalose and lactose by various biochemical methods in the newly developed VERO cell line based Tissue culture anti liquid rabies vaccine.

Key words: Rabies vaccine; Carbohydrate; Trehalose; HPTLC

Introduction

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 (Bloom et al., 2005). 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). Human to human transmission has not been proven. However, some cases of rabies transmission through organ transplantation have been described (Jackson et al., 2006). Since Louis Pasteur's discovery of the rabies vaccine, rabies has been a disease that can be prevented through the timely administration of post-exposure prophylaxis (PEP). Approximately 55,000 people die from rabies each year. The vast majority of these deaths occur in Asia and Africa. Children are at particular risk. Annually more than 10 million people, mostly Asians, receive post exposure vaccination against the dreaded rabies. For the two decades, investigators have been searching for alternative rabies vaccines having all the advantages of the killed or modified live (attenuated) rabies virus vaccines for total stimulation of the immune response in animals and humans, without any undesirable side effects sometimes associated with conventional rabies virus vaccine. Increasing the availability of cell culture rabies vaccines in developing countries is only part of the solution required to reduce the incidence of human rabies. The post exposure prophylaxis requires 4-5 doses of highly expensive cell culture derived vaccines for the entire period of immunization, in which case the poor people are unable to bear the cost. The high cost of cell culture rabies vaccines is due to the increased cost of production. Most of the available cell culture vaccines are in freeze dried state. During processing of the rabies vaccine the maximum cost needed is for the downstream purification as well as for freeze drying. It is very essential to eliminate the impurities as well as to maintain their efficacy and potency.

But during the freeze drying process there is a considerable loss in the antigenicity (immunogenic, potency). The residual moisture content estimation is a mandatory requirement for the analysis of the moisture 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 biomolecule (Greiff, 1971). Residual

moisture reduces the potency and stability of the viral protein. The basic problem in dealing with preservation of viral vaccine is the loss in virus titer either during lyophilization and / 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. Hence during the freeze drying, the viral proteins are supplemented with stabilizers and additives to maintain the stability. Human albumin and Maltose are very common and efficient stabilizers and additives for rabies vaccine (WHO TRS 840).

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 (Crowe et al., 1996). Stabilizers are chemical compounds added to the vaccine and are used in conjunction with either lower temperature storage or lyophilization methods (McAlear and Markus 1979).

The amount of an immunogen that is contained in a vaccine can be extremely small, on the order of tens of micrograms or even 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 vial wall. The types of material that are added to vaccines as stabilizers include sugars such as sucrose, trehalose, maltose, fructose, glucose, sorbitol and lactose amino acids, proteins such as human serum Albumin (HSA) or gelatin (WHO 2006). Carbohydrates are well known reagents to protect bio structure like bacterial and viral protein during freezing and drying

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encountered during the preparation of vaccines. Stabilizers are materials that help to protect the vaccine from adverse conditions such as freeze-drying process. Carbohydrate such as sucrose, lactose, glucose, trehalose and maltose are used as cryoprotectants and as a stabilizer and preservative. The food and drug administration (FDA) approved sugar excipient for parenteral administration and part of the inactive ingredients list or part of FDA approved bio pharmaceuticals are cyclodextrin, fructose, glucose, glycerol, inositol, lactose, maltose, mannitol, sorbitol, sucrose and trehalose. Stabilization of proteins during drying requires a more intimate, hence a more specific interaction between the stabilizer and the test protein (Makino et al., 1991). Only carbohydrates have been shown to be effective in protecting proteins during the drying process (Crowe et al., 1990). Trehalose is unique in that it forms a non-hygroscopic glass that is stable at high temperatures and also when the glass is essentially completely desiccated (Crowe and Crowe, 2000).

Trehalose (α , α -trehalose) is a disaccharide formed by a 1, 1 linkage of two D-glucose molecules. It is a non-reducing sugar that is not easily hydrolyzed by acid and the glycosidic bond is not cleaved by α -glycosidase. The molecular formula and weight are $C_{12}H_{22}O_{11}$ and 342.31 respectively. It is stable under low pH conditions where other disaccharides typically undergo various reactions, such as hydrolysis into their component monosaccharide. Micro needles prepared without trehalose elicited a strong antibody response after just one day of strong but showed lower levels of influenza virus specific Ig-G after 1 week or 1 month storage time. It showed that influenza vaccine loses immunogenicity during storage and that the presence of trehalose can prevent that loss. Microneedles coating with inactivated influenza vaccine reduced vaccine activity and that the addition of trehalose to the coating formulation helped retain immunogenicity (Kim et al., 2010).

Lactose is a sugar that is found most notably in milk. It is a disaccharide that consists of galactose and glucose fragments bonded through a β 1-4 glycosidic linkage. Lactose is also used as stabilizers in some of the viral vaccine formulations (Makino et al., 1991). The currently available cell culture vaccines are formulated with human albumin and maltose as a freeze dried state. There is no any other combination of rabies vaccines with trehalose and lactose as stabilizers of both freeze dried, liquid state. Trehalose, lactose is more superior stabilizer for viral vaccines because increasing immunogenicity was boosted after immunization and also higher stability, lower immunogenicity loses (Kim et al., 2010). Presently a lot of analytical techniques are available for the determination of carbohydrates presents as a food preservative and as a stabilizer in freeze dried, spray dried vaccines. Our study is dealing of the determination of the carbohydrates present in the newly developed liquid rabies vaccine by the utilizing the traditional as well as newer assay techniques.

Materials and Methods

Rabies liquid vaccine

Newly formulated TCALRV-A, TCALRV-C and TCALRV-D (Tissue Culture Anti Liquid Rabies Vaccine) obtained from anti rabies vaccine

production laboratory of The Pasteur Institute of India, Coonoor were used in this study along with its production details as followed Monolayers of VERO cell line inoculated and infected with strains of Pasteur virus (PV11). The virus, viral protein was harvested from infected cultures from the third day after inoculation, five times at the interval of 72hrs each, the virus titers was above the WHO prescribed level ($10^{5.2}$). Subsequently single viral harvest are pooled and concentrated by tangential flow filtration (TFF) using Pellicon system and inactivated by beta propiolactone. Subsequently it was further purified by chromatographic technique. Concentrated, inactivated, purified rabies viral proteins are formulated with stabilizers and additives (Human serum albumin, Trehalose, Lactose and Aluminium Phosphate).

(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.

Preliminary hydrolysis

All the vaccine samples are the process of preliminary hydrolysis for conversion of polysaccharides into monosaccharide was performed by placing 5-10 mg of samples into a glass tube containing 50 ml of 1M HCl. Then that solution was heated at 100°C in a boiling water bath for 120-150 min. After cooling the supernatant was separated by centrifugation and the acid solution was treated with the same procedure used for standard solutions as per the method of Mauro Mecozzi.

Analysis of trehalose

Anthrone method: The amount of trehalose and lactose carbohydrate present in the TCALRV-A, C and D were estimated spectrometrically at 630 nm by anthrone method (Pons et al., 1980). The trehalose converted into two glucose molecules and it was dehydrated to hydroxyl furfural derivatives in the hot acidic medium and gives green colour which was read spectrometrically at 630nm (Hedge and Hofreiter, 1962) 0.2-1.0ml of standard glucose solution with the concentration 20-100 μ g of concentration was taken and the volume was made up 1ml with sterile distilled water. Then add 0.5ml of vaccine samples and made up to 1ml with sterile distilled water. The volumes were added with 4ml of anthrone reagent and it was read at 630nm.

Phenol sulphuric method: Hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This forms a green colored product with phenol and has absorption maximum at 490nm. 0.2-1.0ml of standard glucose with the concentrations varying 20-100 μ g of standard glucose and made up the volumes to 1ml with sterile distilled water. Take 0.1 and 0.2 ml of vaccine samples and make up to 1ml with sterile distilled water. Then add 1ml of phenol solution and add 5ml of 96% sulphuric acid and shake well. Then incubate in water bath for 20minutes at 30°C. The colour development was read at 490nm (Masuko et al., 2005).

Ortho toluidine method: In This Method O- Toluidine reacts in hot glacial acetic acid with the terminal aldehyde group of glucose

S. No	Name	Biomolecules present in Liquid rabies vaccine				
		Purified rabies Viral proteins (%)	BCA protein	Humans Serum Albumin (%)	Trehalose (%)	Lactose (%)
1	TCALRV-A	54.7%	1	5	---	5.99 mg
2	TCALRV-C	60.34%	1	5	---	---
3	TCALRV-D	54.7%	1	2.5	2.5	5.99 mg

Table 1: Liquid rabies vaccine constituent.



Name of the Vaccine	Biochemical analysis- Trehalose											
	Anthrone			Phenol-sulphuric acid			Ortho toluidine			HPTLC		
	EXP.I %	EXP.II %	-	EXP.I %	EXP.II %	-	EXP.I %	EXP.II %	-	EXP.I %	EXP.II %	-
TCALRV-A	6.37	6.41	6.39	6.25	6.21	6.23	4.67	4.73	4.80	4.71	4.81	4.76
TCALRV-C	5.93	5.99	5.96	5.64	5.50	5.57	4.85	4.91	4.88	4.99	4.87	4.93
TCALRV-D*	5.93	5.81	5.87	5.42	5.34	5.3	5.65	5.65	5.65	2.29	2.21	2.25
TCALRV-D	Biochemical analysis- Lactose											
	Anthrone			Phenol-sulphuric acid			Ortho toluidine			Methylamine		
	EXP.I %	EXP.II %	-	EXP.I %	EXP.II %	-	EXP.I %	EXP.II %	-	EXP.I %	EXP.II %	-
	5.93	5.81	5.87	5.42	5.34	5.3	5.65	5.65	5.65	2.41	2.59	2.5

Table 2a: Carbohydrates analysis.

S.No	Vaccine Name	During formulation		Analysis of Carbohydrate			
		Name of the Carbohydrate	% of Carbohydrate	Anthrone [#]	Phenol sulphuric acid [#]	Ortho- Toluidine [#]	HPTLC [#]
1.	TCALRV-A	Trehalose	5 %	6.39 %	6.23 %	4.80 %	4.76 %
2.	TCALRV-C	Trehalose	5 %	5.96 %	5.57 %	4.88 %	4.93 %
3.	TCALRV-D	Trehalose	2.5 %	5.87 %	5.38 %	5.65 %	---
4.	TCALRV-D	Lactose	2.5 %	5.87 %	5.3 %	5.65 %	2.5 % *

*Samples analyzed by Methylamine method

Table 2b: Carbohydrates analysis.

S. No	Name of the Vaccine	Anthrone*	Phenol Sulphuric Acid*	Orthotoluidine*	Methylamine*	HPTLC*
1	TCALRV-A	6.39-5 = -1.39	6.23-5 = -1.23	4.80-5 = +0.20	NA	4.76-5 = -0.24
2	TCALRV-C	5.96-5 = +0.96	5.57-5 = +0.57	4.88-5 = -0.12	NA	4.93-5 = -0.07
3	TCALRV-D	5.87-5 = +0.87	5.3-5 = +0.3	5.65-5 = +0.65	NA	2.29-2.5 = -0.21
4	TCALRV-D	5.87-5 = +0.87	5.3-5 = +0.3	5.65-5 = +0.65	2.5-2.5 = 0	NA

*A-B=C A-Tests average values, B-Formulation Percentage, C-Difference between the average test values and formulation percentage

Table 3: Deviation of carbohydrates in liquid rabies vaccines.

to produce a blue- green colored condensation product measured spectrometrically at 630nm (Fasce et al., 1972). Add 0.1ml of vaccine samples and 0.1- 0.5ml of standard glucose and makeup the volumes to 0.5 ml with sterile distilled water. Then add 5ml of Orthotoluidine reagent and mix well. Close the eppendorf vials and keep in boiling water bath and cool in running tap water. The colour development was read at 630nm (Fasce et al., 1972).

HPTLC method: Trehalose as a standard, n-butanol-pyridine water (8:4:3) as a mobile phase, N-(1-naphthyl-ethylenediamine di hydrochloride in methanol containing 3% sulphuric acid as spraying reagent in the silica gel TLC plate with the absorbance at UV 366nm in linomat 5 wincats 3.1 HPTLC camas analyzer (Ranganathan et al., 2001).

Standardization of impregnate and solvent system for HPTLC of trehalose Preliminary experiments were carried out to find the best combination of impregnate and solvent system for the thin-layer chromatographic separation of trehalose in the presence of other sugars, such as glucose, fructose, maltose, sucrose and raffinose, on silica gel plates (10_10), based on the work of Mezzetti et al. (1971) using TLC. Impregnation was carried out by dipping the silica-gel plates in a solution of the appropriate impregnates for 10 s, the plates were air-dried overnight and activated by keeping at 105 °C for 1 h in an air oven. The sugars were then spotted quantitatively, using a Linomat system; the plates were run once in two different solvent systems in Camag TLC chambers. The solvent systems used included n-propanol: pyridine: water—5:3:2 and n-butanol: pyridine: water—8:4:3. Visualizations of the spots was done by spraying with N- (1-naphthyl)-ethylenediaminedihydrochloride in methanol containing 3% H₂SO₄ (Bounias, 1980). Quantification of trehalose was done using a standard optical density curve at 546 nm against sugar concentration varying from 300 ng to 1 mg/ml).

Lactose analysis

Methylamine method: The lactose standards at different concentration levels were taken and the amount of lactose in TCALRV-D was calculated by methylamine method (Nickerson et al., 1976) at 540nm, Molecular Devices Corporation Sunnyvale, California 94089. With the version of 4.1 software for macintosh® and windows® using 96 well microtitreplate. 0.1 to 0.6 ml each of stock standard lactose and 1ml of vaccine sample put into 2 ml eppendorf vials. 1ml Glycine- NaOH buffer added to all the vials. Further, another 1 ml of glycine NaOH buffer added to each vials. 0.1 ml of methylamine solution and 0.1 ml of sodium sulphite solution added into each tube and mixed thoroughly. Finally all the tubes were incubated in a thermostatically controlled water bath at 65°C for 25 min. Subsequently it was quenched in an ice water bath for 2 minutes. Simultaneously standards similar preparation was made with a 1 ml of Glycine NaOH blank buffer solution. Absorbance measured against blank at 540 nm. The concentration of lactose determined using a standard curve by plotting absorbance against concentration of lactose.

Phenolsulphuric acid method: Using the similar procedure as mentioned for the estimation of trehalose all the liquid vaccine materials were subjected to phenolsulphuric acid method for the residual lactose analysis.

Anthrone method: The anthrone method was done similar to the trehalose method and from this method the residual lactose was analyzed with spectrometrically at 630nm.

Ortho-toluidine method: In this method the lactose was analyzed by O-Toluidine with hot glacial acidic medium and it was read spectrometrically at 630nm.



Results and Discussion

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. All the formulated vaccines potencies are above the WHO recommended level (Table 1). Currently more techniques are available for the estimation of carbohydrate present as a preservative in food products and freeze dried vaccine. So far not much research was done for the estimation trehalose present in the liquid state viral vaccine especially the rabies liquid vaccine. The liquid vaccine samples are further assayed with slight modification of various techniques involved for the determination of carbohydrates present in the freeze dried vaccines. All the experiments are done in duplicates and their means are calculated and tabulated (Table 2a and Table 2b).

When compare with the assay results of trehalose and lactose apart from the HPTLC method all the values of the other methods are above its formulated concentration. The degrees of deviation are +1.39 to +0.65. But the HPTLC deviations are -0.07 to 0.24. In the assay results of the anthrone method the main drawback was is concentrated sulphuric acid which was used as a catalyst. But this method does not give a valid result may be due to its interference with the adjuvant aluminum phosphate in the vaccine. Among many colorimetric methods for carbohydrate determination, the phenol sulphuric acid method is the easiest and most reliable method for measuring neutral sugars in oligosaccharides, proteoglycans, glycoprotein and glycolipids. The modified Phenol-sulphuric acid method for the total carbohydrate estimation has shown to be more accurate than the conventional method.

In case of TCALRV-D, trehalose content was determined by method of anthrone and HPTLC was 5.87% and 2.5% respectively. From the result it is clear that the HPTLC methods can be used as a possible alternative to the other reported methods. In the anthrone method it shows the total carbohydrates, whereas in HPTLC method it shows only the amount of trehalose in the TCALRV-D vaccine.

The presence of proteins often can cause interferences in carbohydrate estimation. In this study, the solution is used in the process and Human serum albumin and purified rabies viral proteins did not interfere in the quantification of total carbohydrate in each sample analyzed. The combination of reducing, non reducing sugars with purified rabies viral proteins in the presence of human serum albumin, aluminum phosphate as additives, adjuvants of liquid rabies vaccine respectively, The TCALRV-A and TCALRV-D only contains Aluminum phosphate as adjuvant, but not in TCALRV-C. In general trehalose is non reducing sugar which does not react with other compounds. But lactose is a reducing sugar; it gets oxidized with available compounds for further reduction. In case of our TCALRV-D there is no oxidation of lactose with other compounds. This may be due to the presence of strong bonds of Human serum albumin and aluminum phosphate. Hydrogen bonds play a role in determining and stabilizing the three dimensional structure of polypeptides.

In that sense determination of carbohydrate in a variety of samples is a basic analytical operation in biotechnological process and a large number of analytical procedures have been developed to measure its presence in water (WHO). Fecal fat plant extract and yeast samples among many colorimetric methods the Anthrone sulfuric acid is one of most commonly used technique.

In the present work, a high performance thin-layer

chromatographic (HPTLC) analysis of trehalose was attempted. In the present study TCALRV -A, TCALRV -C, TCALRV -D was subjected to different analysis (lactose and trehalose). However the production percentage of trehalose is 5.0 % in TCALRV -A, TCALRV-C. In case of TCALRV-D the production percentage of trehalose and Lactose are each 2.5%. But their concentration is above the prescribed level the above menace vaccine samples are further analyzed by HPTLC method and the results are tabulated (Table 3). This technique only revealed more relevant results as well as the deviation of the original production percentage of trehalose, lactose and it ranges from -0.24 to 0.07. Other techniques showed higher deviations than the actual percentage.

Besides, traditional methods for quantifying total carbohydrate are more complex and time consuming. The anthrone, Phenol-sulphuric acid, Orthotoluidine and methylamine methods are described in this paper used for several times in this laboratory and has been found to be suitable to quantify carbohydrates presence in the liquid rabies vaccine production process. In case of lactose analysis, the anthrone, phenol sulphuric acid and ortho toluidine method, the percentage was higher. This is because these analyses show the total carbohydrates and not the amount of lactose. To analyze the lactose amount in these methods a separate test solution of trehalose should be taken with the known concentration and with that result of trehalose, the amount of lactose is calculated by the mean of difference between the total carbohydrates (Lactose and trehalose in vaccine) and the trehalose solution. This is a time consuming process and there are possibilities for error. But in the methylamine method, it shows only the amount of lactose and not the other sugars and it is easier than the above experiments.

Finally the use of HPTLC assay allows total carbohydrate determination in numerous liquid rabies vaccine samples quickly and easily. As well as the trehalose, lactose based liquid state rabies vaccine it may the replacement of freeze dried rabies vaccine, but it requires further study to understand the mechanisms of combinations of reducing and non reducing sugars in the presence of purified rabies viral proteins and human serum albumin and aluminum phosphate.

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