Formulation and Evaluation of Maltose-Leucine-Tetanus Toxoid Dry Powder Inhalation for Pulmonary Delivery

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Abstract: Tetanus toxoid vaccination is primarily used for tetanus prevention. The conventional vaccine for tetanus toxoid is characterised by the limitations of pain, sterility issues, and cold storage. Hence, a pulmonary administered tetanus toxoid (TT) vaccine was fabricated to overcome the limitations of conventional intramuscular vaccines. The goal of this study was to make a dry powder of tetanus toxoid that could be inhaled using maltose and leucine as a vaccine carrier. Additionally, TT dry powder inhalation (DPI) was intended to confer antigenicity and stability. Maltose, leucine and tetanus toxoid were homogenised and centrifuged to obtain the particles of Maltose-leucine-tetanus toxoid (MLTT). Particle size analysis, FTIR, encapsulation efficiency, flocculation, in vitro vaccine release tests, and flow characteristics were all performed on the produced particles. MLTT powder combination remained stable during the process and after storage. FTIR analysis, showed no chemical interactions. The homogenization method produced a powder with the geometrical particle size of the DPI formulation, in the range between 95.6 ± 5.7 nm and 1081.1 ± 10.8 nm which was believed to be appropriate for inhalation. Whereas -27 ± 0.6 mV and 0.496 ± 0.013 were found to be the zeta potential and polydispersity index, respectively. The formation of flocculation of tetanus toxoid with antitoxin as at 20 min confirms the antigenicity of the same. TT was released immediately with 83.2 ± 4.9% within 2 h following the diffusion mechanism. The findings revealed the powder blend's potential as a dry powder inhalation for delivering TT.

Keywords: *Tetanus toxoid, Maltose, Leucine, Dry powder inhalation, Pulmonary administration, Mucosal vaccine.*

1 INTRODUCTION

Tetanus is a life-threatening illness that causes convulsions (seizures) and severe muscle spasms, which can lead to spinal fractures. Tetanus causes death in 30% to 40% of the cases. Tetanus vaccine is recommended for infants aged between 6 and 8 weeks of age and older, as well as for children and adults during minor cuts and surgeries. Additionally, a series of three to four shots is prescribed during maternal care to protect the mother and the neonate. Owing to the short term immunity induced by TT vaccine, immunization for every 10 years is recommended. As can be seen, the implementation of vaccination programmes has been shown to be effective in disease control [2]. Despite the advantages of intramuscular tetanus toxoid vaccination, it has drawbacks such as sterility, discomfort, cold chain storage, and mucosal immunity induction. Mucosal linings are exposed to a wide range of microorganisms and are equipped with innate immune components to prevent microbial penetration [4-9]. Mucosal vaccinations, which are known for their non-invasive approach, are available on the market. Oral, nasal, sublingual, vaginal, and pulmonary vaccine administration routes have all been examined and reported for conferring mucosal and systemic immunity [10-13]. Local mucosal immune responses are becoming more widely recognised as it is important for disease prevention [14]. Vaccines delivered by mucosal ports induced greater mucosal immune response, but vaccines administered via injection are observed to be poor inducers of mucosal immunity and thus less effective against infection invading at mucosal surfaces. [14]. Nonetheless, antigen injection has played a large role in clinical vaccination development, and the majority of vaccinations now in use are administered intramuscularly or subcutaneously. Several findings point to a paradigm shift to mucosal vaccination delivery [15]. Pulmonary administration has a variety of

benefits over alternative delivery routes, including quick drug uptake, a large surface area for solute transport, and good bioavailability, as well as its non-invasive nature, rich vasculature, and low proteinase activity [16]. Furthermore, the production of mucosal antibodies in BAL (bronchial associated lymphoid tissue) fluids has been demonstrated using the pulmonary route [17]. Although the pulmonary tract has numerous advantages, product deposition in the alveoli appears to be difficult. It is worth noting that 0.5-2 µ sized particles have been found to be effective at deposition in the alveoli and small conducting airways due to gravitational sedimentation. In previous research [18], micronization technology was reported to be promising in producing contents suited for deep lung administration. Previous research has shown that homogenization can be used to make micron-sized particles [19]. Metered dosage inhalers (MDIs), soft mist inhalers (SMIs), nebulizers, and dry powder inhalers (DPIs) are all pulmonary products currently on the market [20]. Metered dose inhalers have long been popular due to their simple design and low cost, although there have been reports of inadequate medicine delivery and propellant use. [21]. Soft mist inhalers, on the other hand, are costly, and while nebulisers are commonly used in hospitals, they require periodic heating of the medication, which may damage the heat-sensitive ingredients [11]. Dry powder inhalation is preferred over other inhalation products because of the advantages of medication administration efficiency, the absence of propellants, patient compliance, dose carrying capacity, and the availability of the product form as a solid, which contributes to its stability [11]. At present, DPI is at its budding stage owing to its promising features. Earlier studies have reported that DPI formulations efficiently deliver drugs, peptides, and vaccine constituents for systemic and local action. Hence, DPI is thought to be the best choice for administering tetanus toxoid due to the advantages of the same. The DPI formulations must have the appropriate characteristics for efficient particle deposition in the lungs. Powder physico-chemical properties can be changed using appropriate methodologies and formulation compositions to optimise powder flow and aerosolization [22]. It was discovered that homogenising a powder admixture resulted in micron-sized particles [23]. A DPI is required to exhibit good flow properties for effective deposition of the same. Hence, maltose is selected for the given study owing to its low density and free flowing properties. Maltose is a disaccharide which is commonly used as an excipient in oral and parenteral products [24]. Although the maltose-DPI formulation is expected to exhibit suitable aerodynamic properties, its tendency for aggregation might retard its flow properties. Hence, leucine was included in the formulation to enhance the aerosolization properties of the product by imparting dispersion characteristics to the powder blend. The present investigation focuses on the suitability of maltose-leucine-tetanus toxoid dry powder inhalation formulation for pulmonary administration.

2 METHODS AND RESULTS

2.1 Chemicals and Reagents Used

Maltose was bought from Sigma Aldrich, Bengaluru, India. L-leucine was obtained from Sisco Research Laboratories Pvt. Ltd. Dano Vaccines and Biologicals Pvt. Ltd., Hyderabad, India, generously contributed, with a limit of flocculation (Lf) content of 3000/ml tetanus toxoid and commercial tetanus toxoid, 5 ml vials (each ml contains 5–25 Lf). Tetanus Antitoxin I.P. (3000 I.U/ml) obtained from Premium Serums and Vaccines Pvt. Ltd., Mumbai, India. The rest of the compounds were of analytical grade. The Bradford reagent was provided by Himedia Labs Pvt. Ltd., Mumbai, India.

2.2 Preparation of maltose-leucine-TT DPI formulation

Appropriate amounts of maltose (1.6 g), leucine (2 g) and TT were added to 100 mL of distilled water and mixed for 15 min with a homogeniser (Remi 35L 300-5000 rpm AC/DC Homogeniser and Emulsifier, RQ-130B/D). The preparation was centrifuged (Remi 16 Tubes Centrifuge R8-C) at 1000 rpm for 30 min to separate the product. To get dry powder of the formulation, the supernatant is removed and the sample is dried in a desiccator.

2.3. Maltose-leucine-TT formulation particle size, zeta potential, and polydispersity index

To reach 1% w/v dispersion, the TT formulation was dispersed in de-ionized water and agitated at 100 rpm at 37°C. The particle sizes, zeta potential values, and polydispersity index of different formulations of MLTT nanoparticles were identified using a 532 nm laser and a Zetasizer Nano Instrument (Malvern Instruments, NanoZS, ZEN3600, UK) [22]. Sampling was done in triplicate.

2.4. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra for pure tetanus toxoid and the MLTT formulation as KBr disc were analysed using a Shimadzu 435 U-04 IR spectrophotometer. The sample was optimized, and the peaks that were unique to it were found and recorded [26].

2.5. Determination of flow properties of MLTT powder

The bulk density of a powder is a metric that indicates how light it is. It is necessary for dry powder inhalation to be light and have a low bulk density. It has a high bulk volume and a low bulk density when the particles are loosely packed. Light powders are those that have a low bulk density. A granular substance fills the cylinder. The tapping was repeated 50 times. The original and final volumes after tapping were used to calculate the bulk volume. In the provided formula, the measurements are substituted [37].

$$Bulk\ density = Weight\ of\ powder/Bulk\ volume$$

Once voids or air gaps between particulates are removed, tapped density is often expressed in terms of mass per unit volume. The flow qualities are indicated by the tapped density, which is an essential metric. To determine the same, the powder blend was tapped 1250 times and the volume tapped was measured. In the supplied formula [27], the determined measurements were substituted.

$$Tapped\ density = Weight\ of\ powder/Tapped\ volume$$

The angle of repose determines the DPI's flow characteristic. The flow characteristics are determined by frictional forces. An angle of repose is used to calculate frictional forces. It is the angle formed by the powder heap and the horizontal plane. For this purpose, a suitable amount of the powder blend is permitted to build a pile on the horizontal plane by passing the mixture via a funnel secured to a stand with a clamp. The pile's height (h) and radius (r) are measured, and the angle of repose is calculated using the following formula [27].

$$Tan\theta = h/r$$

2.6. TT encapsulation efficiency

The supernatant was decanted after centrifugation and the TT content was estimated using UV-Visible spectrometry technique [13]. Bradford reagent was utilised in the analysis. To determine the encapsulation efficiency, a standard curve and the following equation were used [28].

$$\%EE = TT\ entrapment/TTtotalX100$$

2.7. Estimation of TT content

A suitable amount of the given sample of MLTT was transferred to a mortar, distilled water was added, and the sample was triturated to 1 mg/ml. A UV-Visible spectrophotometer (Labindia 3000+ UV-Visible spectrophotometer) was used to analyse 30 µl of sample with 1.5 ml of Bradford reagent at 295 nm [29].

2.8. Flocculation test

The flocculation test was used to determine the structural integrity of the loaded TT. In this method, suitable dilutions of toxoid under test were pipetted out into a series of round bottom tubes. The MLTT equivalent to 50 Lf was weighed and transferred to a series of six flocculation tubes. Antitoxin was added in graduated dosages differing by 10% to these flocculation tubes in volumes, and the mixtures were incubated at 50 ± 1°C. The Lf value of a toxoid is determined by the flocculation period and antitoxin concentration at which the toxoid's initial flocculation was observed following incubation. The time duration of the flocculation (Kf) was tracked and noted. This can be used to determine the toxin's quality. If the Kf is low, the antigen quality is good, and vice versa [30, 31].

2.9. In vitro TT release studies

The optimised MLTT formulation was placed on a filter paper between the donar and receiver compartments of a Franz diffusion cell apparatus containing pH 7.4 phosphate buffer saline to imitate the circumstances in the pulmonary area [13]. The apparatus is placed on a magnetic stirrer and whirled continuously for 4 h. At regular intervals, samples were taken and analysed using UV-Visible spectrophotometry at 295 nm with Bradford reagent.

2.10. Stability studies

The stability studies were conducted to check the effect of storage conditions and shelf life on the prepared formulation. These tests were done for MLTT samples in air tight vials kept at 4°C/0% RH and 25 °C/60% RH for a year, and the flocculation test as described in section 2.9 was used to measure how stable the samples were.

3 DISCUSSION

3.1. Measurement of particle size

Although particles in the 1-5 µ range have been found to have excellent deposition properties for deep lung delivery, particles in the nanometric range result in deposition at the deeper sites of the respiratory tract. A Malvern Zetasizer was used to determine the geometrical particle size of the DPI formulation, which was found to be in the range between 95.6 ± 5.7 nm and 1081.1 ± 10.8 nm. Due to the submicron size ranges of polymer particles, microspheres with a diameter smaller than 2 µm will have even better cellular absorption [31]. The volume weighted median, d (0.5), for the geometric particle size distribution was 233.8 ± 5.9 nm. The size of 10% volume of the particles with a diameter below d (0.1) was 108 ± 9.8 nm and of 90% having a diameter below d (0.9) was 1081.1 ± 10.8 nm.

Earlier studies reported that smaller size particles, below the micron range, have a lot of free energy and tend to congregate. They have low flowability and aerosolization performance, and they tend to cling to the inhaler. However, the incorporation of L-leucine can enhance the aerosolization. Further, L-leucine has previously been employed in DPI formulations and possesses anti-adhesive qualities as well as the capacity to disrupt particle-particle interactions [33]. The powder containing L-leucine was less aggregated and seemed less dense than the other formulations on a macroscopic level [34]. Hence in the present investigation, we have used L-leucine to reduce particle adhesion. The zeta potential and polydispersity index were determined to be -27 ± 0.6 mV and 0.496 ± 0.013 respectively. Because of the presence of repulsive forces, the negative charge of the particulates is thought to hinder the formation of aggregates. Particle size distribution is crucial for aerosol quality and efficiency [34] and the polydispersity index (PDI) is widely used to assess it. A greater carrier PDI value indicates a wider particle size distribution, resulting in a more heterogeneous drug combination. As a result, medication deposition in the lungs following inhalation may be more variable [35]. The PDI of 0.496 suggests that the dispersion was homogeneous.

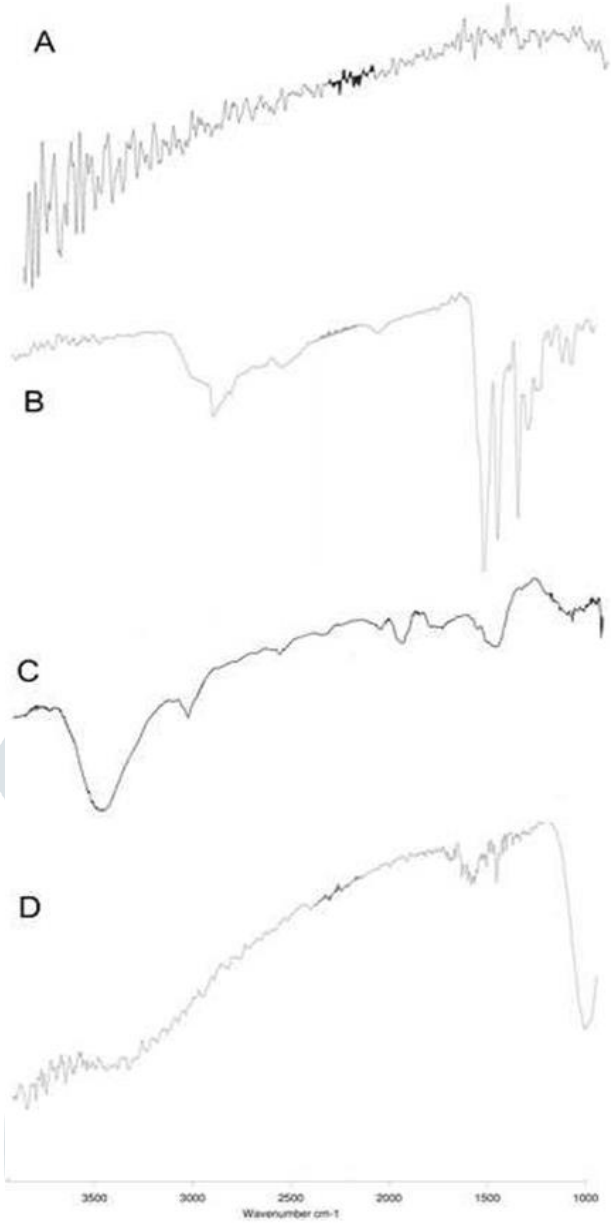

Figure: 1 *FTIR spectra of (A) maltose; (B) leucine; (C) tetanus toxoid; and (D)maltose-leucine-TT formulation*

3.2. Fourier transform Infrared spectroscopy (FTIR)

The FTIR spectra of pure TT and TT formulation are shown in Fig. 1, with peaks at 3416,1646.2, and 629.6 cm-1 for pure TT and similar peaks at 3400 and 1600 cm-1 for the formulation. The findings indicated that the tetanus toxoid was not damaged during the preparation.

3.3. Flow properties, encapsulation efficiency and TT content

The performance of the MLTT DPI formulation is critically impacted by the particle size and flow properties of the same. During breath actuation, the dry powder inhalations should have adequate flow qualities to deagglomerate and disperse for efficient deposition. The flow parameters of the powder blend are characterized by angle of repose, bulk density, and tapped density. For good flow characteristics, the parameters should report lower values of angle of repose and bulk density. As shown in Table 1, the formulation of maltose-leucine and tetanus toxoid dispersion has good flow characteristics. The suggested formulation's encapsulation efficiency was found to be 96.1 ± 4.51%. The drug content of the sample was reported as 93.8 ± 3.27%.

Table 1 *Determination of flow properties*

Parameters	Formulation
Angle of repose (θ)	16.6 ± 1.42

Bulk density g/cm^3	0.625 ± 0.02
Tapped density g/cm3	0.833 ± 0.01

3.4. Flocculation test

The WHO Expert Committee on Biological Standardization established that flocculation tests utilising reference toxoids are highly reproducible and trustworthy [26]. The flocculation test indicates the stability and antigenicity of the sample. The formation of flocculation of tetanus toxoid with antitoxin as seen in the tubes which confirms the antigenicity of the same. Additionally, the flocculation time observed implies the quality and stability of the toxoid in the formulation. In the given study, flocculation occurred after 22 ± 1 min, implying the viability of TT in the prepared MLTT formulation.

3.5. TT release studies in vitro

The Franz diffusion cell apparatus was used for the in vitro TT release studies from MLTT formulation. TT was released immediately, as shown in Fig. 2 and 83.2 ± 4.9% of the drug was released within 2 h. The findings showed that maltose and leucine had no effect on the release of tetanus toxoid. Furthermore, the correlation coefficient of r^2 (0.9717) revealed that the TT release followed first-order kinetics. The r^2 value of the Higuchi plot (0.936) revealed that the TT release from the MLTT formulation was diffusion controlled.

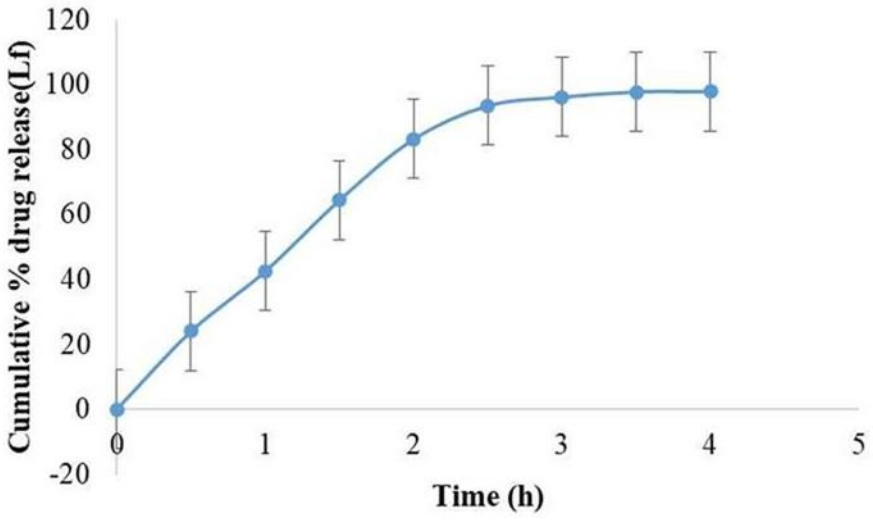

Figure 2 *Diffusion profile of maltose-leucine-TT DPI*

3.6. Stability studies

The stability of the dry powder inhalation of the MLTT formulation was observed by the flocculation test, and the flocculation was observed at 20 ± 0.5 min. The study indicated the stability of the vaccine at 25°C/60% RH without the necessity of refrigerating conditions for 12 months. There was no change in the results of product stored at 4°C/0% RH. As a result, the tetanus toxoid dry powder inhalation can be stored at room temperature without the need for cold chain storage. Furthermore, the findings suggest the commercialization potential of the dry powder vaccine.

4. CONCLUSION

The present investigation focuses on the finding of maltose-leucine-tetanus toxoid dry powder inhalation formulation for pulmonary administration without without cold chain storage. In the present study, L-leucine was employed in the preparation of the formulation to impart anti-adhesive qualities as well as the capacity to disrupt particle-particle interactions. The DPI formulation showed that the maltose-leucine combination could be a promising carrier system for delivering tetanus toxoid to the respiratory tract owing to its suitable flow properties and stability features.

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