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Development and Characterization of Glutathione Lyophilized Injection

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ABSTRACT

The main objective of present research work was to design, development and characterization of glutathione lyophilized injection. Component excipients were selected based on the preliminary studies. Solubility trials were done to determine the solubility of Glutathione in water with different concentration of sodium bicarbonate. Then glutathione injection was formulated and lyophilized by setting the lyophilization cycle parameters. But, when glutathione was lyophilized alone the cake collapse was observed. So, in order to overcome this problem lyoprotectants (mannitol, lactose, trehalose and sucrose) were added in different concentration i.e. 5% and 10%. The lyophilization cycle was developed for these formulations by changing the process parameter. After formulation development, lyophilization cycle was optimized by reducing the total cycle time. Cycle time reduced at the initial stage of primary drying till the formulation remained stable. First stage of primary drying was reduced to 2520 minutes where the cake remains elegant. The batch L1 developed by FD cycle 11 with 5% trehalose having total lyophilization cycle time 59.75 hours was considered as optimized formulation because it exhibited a good cake formation and pH, moisture content, reconstitution time and assay was found within the range of desired product profile. Short term stability studies were conducted for the optimized formulation as per ICH guidelines for a period of 30 days which revealed that the formulation is stable. It was conclude that the lyophilization technique proves to be an advantage for development of stable injectable dosage form of Glutathione.

Keywords: Glutathione, Lyophilized Injection, Injectable Dosage Form.

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INTRODUCTION

Freeze-drying or lyophilization is a multi-stage operation of effectively drying a material while preserving its biological properties. The material is subject to being frozen and sublimated under vacuum upon heating. Lyophillized products are more stable, easily transported and quickly reconstituted. A benefit of low-temperature operation envisages its applications in pharmaceuticals for heat-sensitive components including vaccine and biological products. [1-5]

The most common mechanism of instability in parenteral formulations is hydrolysis. Regardless of whether the formulation is a true solution, co-solvent solution, emulsion, or contains a complexing agent, the largest component of the formulation is likely to be water. Frequently, the only formulation strategy that will result in adequate stability is water removal. This is usually achieved by means of lyophilization. Lyophilization has a number of advantages over other potential drying methods, such as the ability to obtain an elegant end product with very low moisture content, and significantly, the fact that it is amendable to being carried out in an aseptic environment. [6-7]

The main principle involved in freeze drying is a phenomenon called sublimation, where water passes directly from solid state (ice) to the vapor state without passing through the liquid state. Sublimation of water can take place at pressures and temperature below triple point i.e. 4.579 mmHg and 0.0099 °C. Lyophilization is carried out below the triple point to enable conversion of ice into vapor, without entering the liquid phase. [8-13]

Lyophilization is the most common method for manufacturing of pharmaceutical products that have to be dried thoroughly in order to ensure stability. The stability of the drug during the process and storage and duration of the cycle are two major considerations for optimization of freeze drying process. [14-16]

Reduced glutathione (GSH) is the most prevalent non-protein thiol in animal cells. Its de novo and salvage synthesis serves to maintain a reduced cellular environment and the tripeptide is a co-factor for many cytoplasmic enzymes and may also act as an important posttranslational modification in a number of cellular proteins. The cysteine thiol acts as a nucleophile in reactions with both exogenous and endogenous electrophilic species. As a consequence, reactive oxygen species (ROS) are frequently targeted by GSH in both spontaneous and catalytic reactions. [17-20]

Glutathione (GSH) is taken as therapeutic agent which is water-soluble tripeptide composed of the amino acids glutamine, cysteine, and glycine. The thiol group is a potent reducing agent, rendering GSH the most abundant intracellular small molecule thiol, reaching millimolar concentrations in some tissues. As an important antioxidant, GSH plays a role in the detoxification of a variety of electrophilic compounds and peroxides via catalysis by glutathione S-transferases (GST). [21-25]

Pharmaceutical companies often use freeze-drying to increase the shelf life of products, such as vaccines and other injectables. Lyophilized products possess advantages such as enhanced product stability in dry state, rapid reconstitution time and ease of processing a liquid, which simplifies aseptic handling. [26-30]

The objective of the present research is to formulate and evaluate a lyophilized preparation of glutathione for enhanced stability and therapeutic efficacy. [31-32]

MATERIALS AND METHOD

Chemicals

L-Glutathione reduced was purchased from Kyowa Hakko Bio Co., Ltd. (Japan). Mannitol (Pharmaceutical grade) and Lactose (Pharmaceutical grade) was purchased from Roquette Riddhi Siddhi Pvt. Ltd. (Ahmedabad, India). Trehalose (Injectable grade) was purchased from Hayashibara Co. Ltd. (Japan). Sucrose (Pharmaceutical grade) was purchased from Global calcium Pvt Ltd.. Sodium bicarbonate (Pharmaceutical grade) was purchased from Merck KGaA. Other reagents and chemicals were of analytical grade.

Method

Identification of Drug:

Identification of drug is first step in research methodology. The authenticity of drug was identified by FT-IR, DSC and X- ray powder diffraction analysis.

DSC Analysis:

Thermal behaviour of drug was examined using thermal analyser. An accurately weighed sample was placed in sealed aluminium pans before heating at a scanning rate of 10 °C per minute from 25 °C to 300 °C. An empty aluminium pan was used as reference.

FT-IR Analysis:

The FT-IR spectrum of obtained sample of drug compared with standard FT-IR specta of pure drug. IR spectra of drug was taken by using KBr pellet method. 1 mg drug and 100 mg KBr i.e. (1:100) were accurately weighed and mixed in pestle motor, and discs were formed using a pellet press. The discs were then placed and analysed in FT-IR spectrophotometer.

Solubility:

Solubility of drug was determined in water. An excess quantity of drug was added in 1 ml of solvent in screw capped glass test tube and shaken for half an hour until equilibrium was attained at room temperature.

X- Ray diffraction of pure glutathione:

The X-Ray diffraction of glutathione was done using Scintaz X-ray powder diffractometry instrument, round standard sample holder with round zero background plate, with a cavity of (diameter) 0.5 (dpt) mm and the scanning parameter were fixed in the range of 10-60º two theta in continuous scan mode with step size and rate of 0.05 degree and 3 degree/min respectively.

Drug excipient compatibility study

Compatibility must be established between the active ingredients and other excipients to produce a stable, efficacious and safe product. Here FT-IR and DSC analysis of Glutathione with lyoprotectants and other excipients was undertaken to assess the compatibility of drug with the excipients and to ascertain that there is no interaction between drug and the excipients.

DSC for determination of glass transition temperature

DSC, is a technique that is well suited to determine a complete physic-thermal profile include the physical state of the materials in the frozen state (crystalline, amorphous, metastable, mixed) and the critical temperatures associated with those different phases (glass transition temperature, eutectic temperature and collapse temperature) Temperature: equilibrate at -50.0 ºC, Nitrogen flow: 50 m/min, Heating rate: 5 ºC/min, Sample quantity: 20 µl, Pan: Hermetically sealed aluminum pan, Sampler: auto sampler.

Assay: Assay was determined by HPLC method.

- **Mobile phase:** Prepare filtered and degassed mixture of 30 volumes of methanol and 970 volumes of buffer preparation.
- **Diluent:** Use mobile phase as diluent.
- **Standard preparation:** Accurately weigh and transfer about 60 mg of glutathione working standard into 100 ml volumetric flask. Add about 70 ml of diluent and sonicate to dissolve. Cool and dilute to volume with diluent and mix. Further dilute this solution 5 ml to 25 ml with diluent.
- **Test preparation:** Take five vials of injection and reconstitute each with 5 ml of water, mix all containers. Further weight accurately about equivalent to 240 mg of glutathione (about 2 ml) of this solution into 200 ml volumetric flask and make up to the mark with diluent, mix well. Further pipette out 5 ml into 50 ml volumetric flask and make up to the mark with diluent, mix well.
- **Chromatographic parameters(31):** The liquid chromatography system included a UV detector, injector, and data processor. A Hypersil BDS C18 column (250 mm × 4.6 mm, 5 µm) was used for the analysis. The detection wavelength was set at 210 nm, and the flow rate was maintained at 1 mL/min. A sample volume of 20 µL was injected for each run. The total run time was 7 minutes, while the retention time of the drug was observed at around 5 minutes.

- **Procedure:** Inject single injection of diluent and duplicate injection of test preparation into liquid chromatograph and record chromatograms. Measure response of major peak for assay.

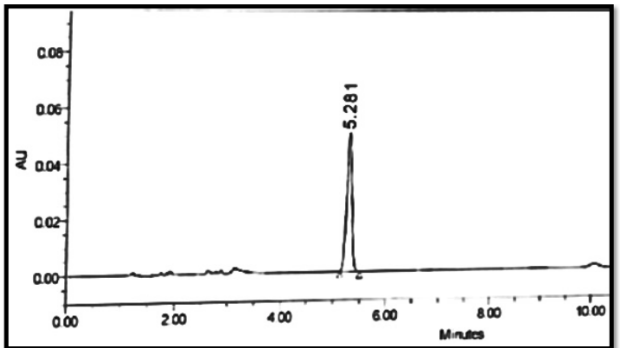

Figure –1: Chromatogram of glutathione

Calculation:

Glutathione (mg/ml) = $\frac{Au \times W_1 \times 5 \times 200 \times 50 \times W_3 \times P}{As \times 100 \times 25 \times W_2 \times 5 \times 100}$

Where,

Au = Average area of Glutathione peak obtained from duplicate injections of assay preparation.

As = Average area of Glutathione peak obtained from five replicate injections of standard preparation.

W1 = Weight of Glutathione standard, in mg.

W2 = Weight of sample taken, in mg.

W3 = Weight per mL of sample, in mg/mL.

P = Potency of Glutathione standard in %, on as is basis.

Calculate the % Glutathione using the following formula:

% of Glutathione = $\frac{\text{Assay in mg/ ml x 100}}{\text{Label claim}}$

Method of Preparation of Glutathione Lyophilized Injection:

Glass vials and slotted rubber stoppers were washed and dried properly. Weighed and dispensed all material accurately. Glutathione were added to WFI and stir to produce dispersed solution of drug. Then sodium bicarbonate were added gradually to the dispersed solution of drug to dissolve drug completely then lyoprotectants i.e. mannitol, lactose, trehalose and sucrose were added under stirring. Then volume were adjusted with WFI and pH of solution was noted down. Then filtered the bulk solution through Syringe filter PVDF (0.22 µm) to get sterile solution and filled into 5 ml glass vials. Then filled vials were partially stoppered using slotted rubber stoppers and then were placed on shelves in lyophilizer. Temperature probe was set in one of the vial to check the product temperature during lyophilization cycle. Lyophilization cycle was developed by varying the process parameter shown in Table 2 to Table 13 such as shelf temperature, chamber pressure and time in

consideration of glass transition temperature and collapse temperature. After completion of lyophilization cycle vials were completely stoppered within the chamber to maintain sterile condition then finished product sealed and labelled properly.

Preliminary study: Effect of lyoprotectant on lyophilization

Lyophilization is best suited when least excipients are used. Initially Glutathione alone was lyophilized and then further on the basis of results (visual inspection) different excipient were used i.e. mannitol, lactose, sucrose and trehalose in 5% and 10 % concentration as shown in Table 1 to study the significant effect on the visual appearance and physical characteristic property of product.

Formulation of Preliminary batches:

Table 1: Formulation of Trial Batches

Sr. no.	Trials	Formulation (quantity per vial)						
		API (mg)	Sod. Bi-carbonate (mg)	Mannitol	Lactose	Trehalose	Sucrose	WFI (ml)
1	A1	600	160	-	-	-	-	q.s to 4
2	A2	600	160	-	-	-	-	q.s to 2
3	M1	600	160	5%	-	-	-	
4	M2	600	160	10%	-	-	-	
5	L1	600	160	-	5%	-	-	
6	L2	600	160	-	10%	-	-	
7	T1	600	160	-	-	5%	-	
8	T2	600	160	-	-	10%	-	
9	S1	600	160	-	-	-	5%	
10	S2	600	160	-	-	-	10%	

Development of Lyophilization cycle for Trial Batches:

The sterile 5 ml USP Type I, glass vial and grey bromo butyl rubber stopper were employed for storage of formulation throughout the process there for the diameter of vial taken as constant for all trial batches. The fill volume also taken constant that was 2 ml for all the batches except A1. The temperature for freezing and sublimation was decided on the basis of glass transition temperature and collapse temperature. Lyophilization cycle consist of three stages (a.) freezing, (b.) primary drying and (c.) secondary drying and the variation in process parameter for preliminary trials have shown in Table 2 to Table 13.

FD1. Lyophilization Cycle for A1 & A2 batch:

Table 2: Lyophilization Cycle for A1 & A2 Batch

Stages of lyophilization	Step	Temperature (ºC)	Time (min)		Vacuum (mT)	
			Rate	Hold	Rate	Hold
Freezing	1	-15	35	30	-	-
	2	-45	30	240	-	-
Primary drying	1	**-30**	**15**	2880	250	200

	2	-25	5	60	175	150
	3	25	50	60	100	75
Secondary drying		30	300		50	
Total time			61.75 (hours)			
Inference	**Complete collapse of cake in F1A and partial collapse in F1 B**					

FD2. Lyophilization Cycle for A2 batch:

Table 3: Lyophilization Cycle for A2 Batch

Stages of lyophilization	**Step**	**Temperature (ºC)**	**Time (min)**		**Vacuum (mT)**	
			Rate	Hold	Rate	Hold
Freezing	1	-15	35	30	-	-
	2	-45	30	240	-	-
Primary drying	1	**-35**	**10**	2880	250	200
	2	-25	**10**	60	175	150
	3	25	50	60	100	75
Secondary drying		30	300		50	
Total time			61.75 (hours)			
Inference	**Partial collapse of cake and cracks on surface**					

FD3. Lyophilization Cycle for A2 batch:

Table 4: Lyophilization Cycle for A2 Batch

Stages of lyophilization	**Step**	**Temperature (ºC)**	**Time (min)**		**Vacuum (mT)**	
			Rate	Hold	Rate	Hold
Freezing	1	-15	35	30	-	-
	2	-45	30	240	-	-
Primary drying	1	-30	15	2880	250	200
	2	**-15**	**50**	60	175	150
	3	25	10	60	100	75
Secondary drying		30	300		50	
Total time			61.75 (hours)			
Inference	**Melt back of cake**					

FD4. Lyophilization Cycle for A2 batch:

Table 5: Lyophilization Cycle for A2 Batch

Stages of lyophilization	**Step**	**Temperature (ºC)**	**Time (min)**		**Vacuum (mT)**	
			Rate	Hold	Rate	Hold
Freezing	1	-15	35	30	-	-
	2	-45	30	240	-	-
Primary drying	1	**-35**	**10**	2880	250	200
	2	-25	10	60	175	150
	3	15	40	60	100	100
	4	25	10	60	75	75
Secondary drying		30	300		50	
Total time			**62.75 (hours)**			
Inference	**Shrinkage of cake**					

FD5. Lyophilization Cycle for M1, M2, L1, L2, T1, T2, S1 and S2 batch:

Table 6: Lyophilization Cycle for M1, M2, L1, L2, T1, T2, S1 and S2 batch

Stages of lyophilization	**Step**	**Temperature (ºC)**	**Time (min)**		**Vacuum (mT)**	
			Rate	Hold	Rate	Hold
Freezing	1	-15	35	30	-	-
	2	-45	30	240	-	-
Primary drying	1	**-35**	**10**	2880	250	200
	2	-25	10	60	175	150
	3	15	40	60	100	100
	4	25	10	60	75	75
Secondary drying		30	300		50	
Total time			**62.75 (hours)**			
Inference	**Cake appearance is good for lyoprotectant as trehalose 5% as compare to other**					

FD6. Lyophilization Cycle for L1 batch to reduce moisture:

Table 7: Lyophilization Cycle for L1 batch to reduce moisture

Stages of lyophilization	**Step**	**Temperature (ºC)**	**Time (min)**		**Vacuum (mT)**	
			Rate	Hold	Rate	Hold
Freezing	1	-15	35	30	-	-
	2	-45	30	240	-	-
Primary drying	1	**-35**	**10**	2880	250	200
	2	-25	10	60	175	150
	3	15	40	60	100	100
	4	25	10	60	75	75
Secondary drying		30	**480**		50	
Total time			**65.75 (hours)**			
Inference	**Again, Good cake with elegant appearance obtained for lyoprotectant as trehalose 5%**					

Optimization of lyophilization cycle: After formulation development process optimization was done by reducing the primary drying time of lyophilization cycle. Lyophilization cycle was reduced at the first stage of primary drying and kept reducing till melt back or collapse occurred. the process parameters for optimization of lyophilization cycle were taken as shown in Table 8 to Table 13.

FD7. Lyophilization Cycle for process optimization:

Table 8: Lyophilization Cycle for process optimization

Stages of lyophilization	**Step**	**Temperature (ºC)**	**Time (min)**		**Vacuum (mT)**	
			Rate	Hold	Rate	Hold
Freezing	1	-15	35	30	-	-
	2	-45	30	240	-	-
Primary drying	1	-35	10	**1440**	250	200
	2	-25	10	60	175	150
	3	15	40	60	100	100
	4	25	10	60	75	75

Secondary drying		30	480	50
Total time			**41.75 (hours)**	
Inference	**Melt back of cake**			

FD8. Lyophilization cycle for process optimization:

Table 9: Lyophilization cycle for process optimization

Stages of lyophilization	**Step**	**Temperature (ºC)**	**Time (min)**		**Vacuum (mT)**	
			Rate	Hold	Rate	Hold
Freezing	1	-15	35	30	-	-
	2	-45	30	240	-	-
Primary drying	1	-35	10	**2160**	250	200
	2	-25	10	60	175	150
	3	15	40	60	100	100
	4	25	10	60	75	75
Secondary drying		30	480		50	
Total time			**53.75 (hours)**			
Inference	**Slight collapse of cake**					

FD9. Lyophilization cycle for process optimization:

Table 10: Lyophilization cycle for process optimization

Stages of lyophilization	**Step**	**Temperature (ºC)**	**Time (min)**		**Vacuum (mT)**	
			Rate	Hold	Rate	Hold
Freezing	1	-15	35	30	-	-
	2	-45	30	240	-	-
Primary drying	1	-35	10	**2760**	250	200
	2	-25	10	60	175	150
	3	15	40	60	100	100
	4	25	10	60	75	75
Secondary drying		30	480		50	
Total time			**63.75 (hours)**			
Inference	**Good cake**					

FD10. Lyophilization cycle for process optimization:

Table 11: Lyophilization cycle for process optimization

Stages of lyophilization	**Step**	**Temperature (ºC)**	**Time (min)**		**Vacuum (mT)**	
			Rate	Hold	Rate	Hold
Freezing	1	-15	35	30	-	-
	2	-45	30	240	-	-
Primary drying	1	-35	10	**2640**	250	200
	2	-25	10	60	175	150
	3	15	40	60	100	100
	4	25	10	60	75	75
Secondary drying		30	480		50	
Total time			**58.15 (hours)**			
Inference	**Good cake**					

FD11. Lyophilization cycle for process optimization:

Table 12: Lyophilization cycle for process optimization

Stages of lyophilization	Step	Temperature (ºC)	Time (min)		Vacuum (mT)	
			Rate	Hold	Rate	Hold
Freezing	1	-15	35	30	-	-
	2	-45	30	240	-	-
Primary drying	1	-35	10	**2520**	250	200
	2	-25	10	60	175	150
	3	15	40	60	100	100
	4	25	10	60	75	75
Secondary drying		30	480		50	
Total time			**59.75 (hours)**			
Inference	**Good cake**					

FD12. Lyophilization cycle for process optimization:

Table 13: Lyophilization cycle for process optimization

Stages of lyophilization	Step	Temperature (ºC)	Time (min)		Vacuum (mT)	
			Rate	Hold	Rate	Hold
Freezing	1	-15	35	30	-	-
	2	-45	30	240	-	-
Primary drying	1	-35	10	**2400**	250	200
	2	-25	10	60	175	150
	3	15	40	60	100	100
	4	25	10	60	75	75
Secondary drying		30	480		50	
Total time			**57.75 (hours)**			
Inference	**Slight collapse**					

EVALUATION PARAMETERS:

Visual examination:

The lyophilized vial was examined for defects in product cake appearance such as optical cake structure, chimney, foam, crust or glaze, ring formation, shrinkage, cracking, total and partial collapse, total and partial melt back, browning and poor shelf supporting structure.

Reconstitution time:

The lyophilized vials of formulation was reconstituted with 5 ml water for injection. The time required for formation of clear solution is note down.

pH of reconstituted solution:

The lyophilized formulations were reconstituted with 5 ml water for injection and the pH of the reconstituted solution was checked.

Residual moisture:

Residual moisture was calculated using auto Karl Fischer Titration. Reagent was added to burette. Sample solvent to the titration vessel was added. Vessel then stirred; the instrument was zeroed by

titrating unwanted moisture in the system. Weighed sample to the titration vessel then added. Reagent then added from the burette while stirring. When the end point was reached, the electrode were detected no change upon addition of more reagents. By knowing how much titrant wad added, auto titrator calculates the water content and reports the result as "percentage water" which should be within 5% is acceptable criteria.

Percentage transmittance:

It was determined by using UV Spectrophotometer. The wavelength was set to 650 nm and instrument was auto zero and then percentage transmittance of the test solution was measured against blank.

Reconstitution stability:

To check the stability of final lyophilized formulation after reconstitution, reconstitution stability is carried out. 10 vials of lyophilized formulation was taken and each vial was reconstituted with WFI and observed visually for any loss of transparency and clarity after every 1 hour for period of 8 hours.

Percentage Drug

Content: Assay was carried out using HPLC according to USP and the results were reported.

Short Term Accelerated Stability Study:

Stability is defined as the extent to which a product retains, within its specified limits and throughout its period of storage and use (i.e. its shelf life), the same properties and characteristics that it possessed at the time of its manufacture. Stability testing is performed to ensure that drug products retain their fitness for use until the end of their expiration date. Short term accelerated stability study as per ICH guidelines was carried out for final formulation at 40 ± 2 ℃ temperature and 75 ± 5% RH for a period of 30 days and sample were analysed for visual appearance, pH, moisture content, percentage absorbance, percentage transmittance, assay and reconstitution time.

RESULTS AND DISCUSSION

Identification of Drug:

DSC Analysis:

As shown in Figure 2 the DSC thermogram of Glutathione showed endothermic peak at 199.78 °C corresponding to its melting point. The onset of melting was observed at 196.48 °C and endset at 204.58 °C. Melting point was nearly same as reported in literature which accounted for purity and identity.

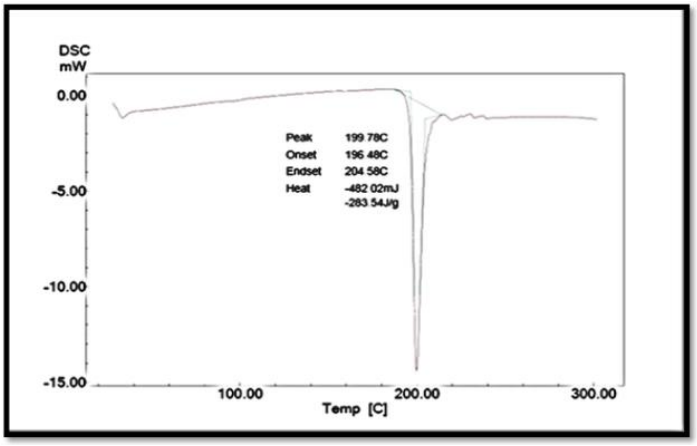

Figure 2: DSC Thermogram of Glutathione.

FT-IR Spectral Analysis: The IR spectrum of sample drug glutathione and the reference spectrum given in Japanese pharmacopoeia are shown in Figure – 3 and Figure – 4 respectively was found to be similar. The Table 14 shows all the characteristic peaks of glutathione found in both the spectrum and functional groups assigned in the wave number exhibited same wave length and had similar intensities to that of the reference spectrum.

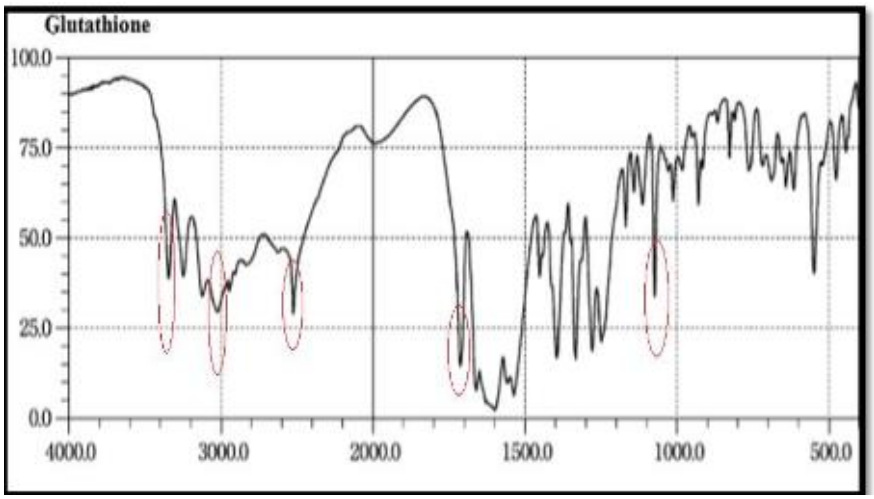

Figure 3: FT-IR Spectrum of Reference Standard Glutathione.

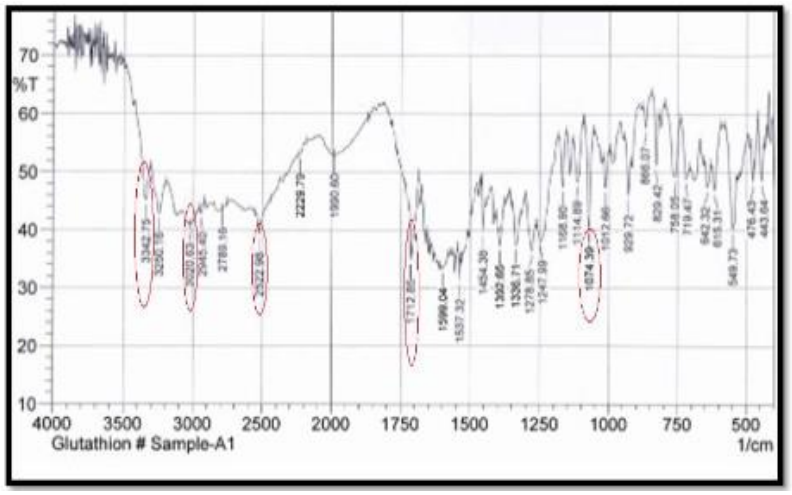

Figure 4: FTIR Spectrum of Pure drug sample Glutathione.

Table 14: Comparison of FTIR Spectrum of drug sample and reference standard

Sr. No.	Functional Groups	Peaks of Functional Groups (cm^{-1})	
		Reference Standard	Drug Sample
1	N-H (1° Amine) Stretch	3340	3342.75
2	O-H	3025	3020.63
3	C=O	1720	1712.85
4	S-H	2550	2522.98
5	C-N	1020	1074.39

Solubility: The solubility of Glutathione was determined in water and it was found 150 mg/ml.

X- Ray diffraction Analysis:

The X- ray diffraction crystallography of pure Glutathione and Glutathione lyophilized injection was done using scintaz, X- ray powder diffractometry in the range of 20-60º two theta, 0-6000 cps and from the XRD pattern it was concluded that Glutathione was found crystalline in nature and various peaks were shown in Figure – 5.

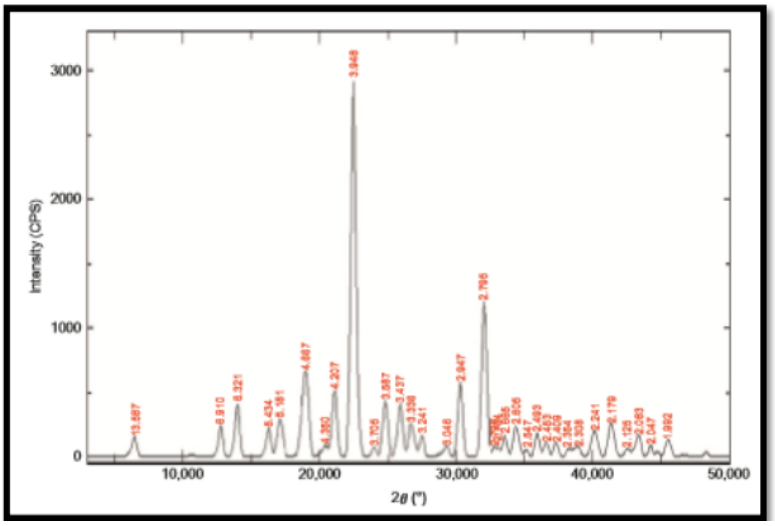

Figure 5: XRD pattern of pure Glutathione

Drug-Excipient Compatibility Study:

DSC analysis: From study of DSC thermograph of pure drug and physical mixture shown in Figure – 6 and Figure – 7 respectively, it was found there was no significant effect on endothermic peak of drug. So, there was no interaction between drug and excipient and all are compatible to each other.

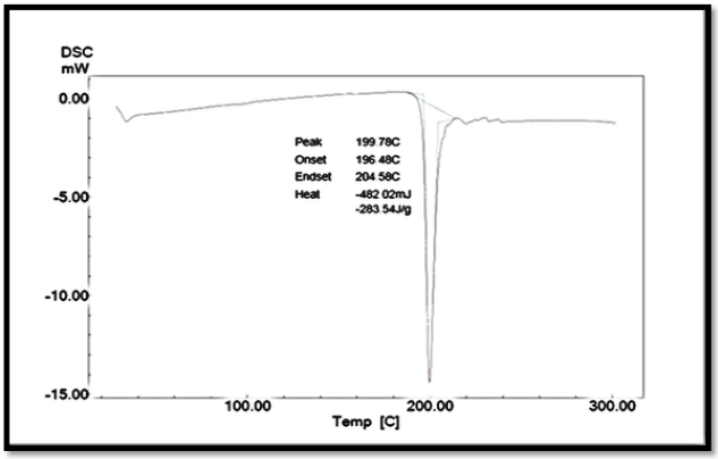

Figure 6: DSC Thermogram of Glutathione

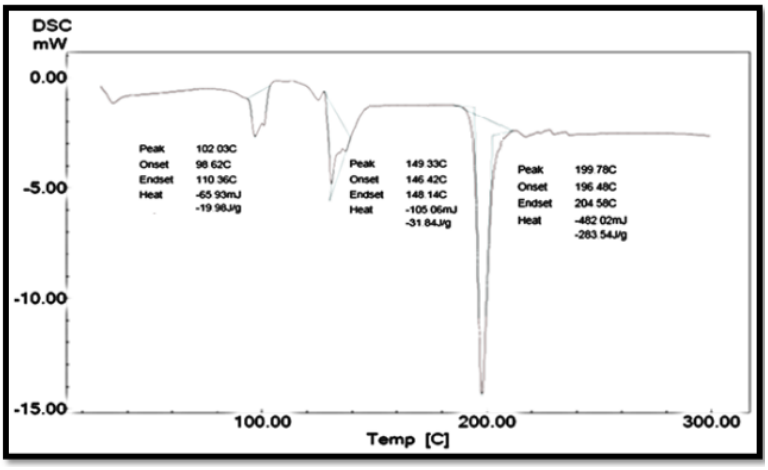

Figure 7: DSC Thermogram of Physical Mixture of Drug and all the Excipient (Sodium bicarbonate and Trehalose)

FT-IR Analysis:

Apart from DSC study, FT-IR study was also performed to determine drug-excipient compatibility. From the study of FT-IR of pure drug and physical mixture of drug with excipients. From the observation and identification of peaks, it was found that there was no significant interaction between drug and excipient and all are compatible to each other.

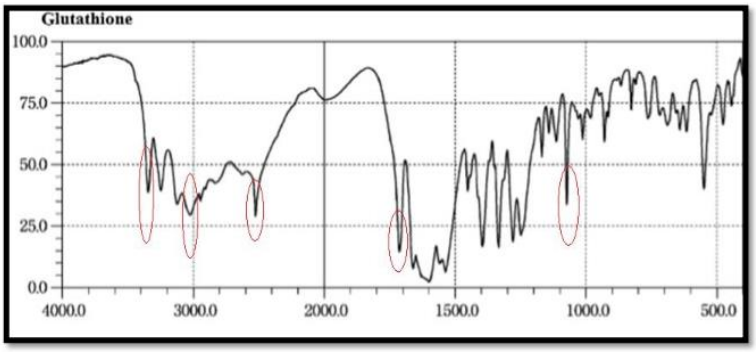

Figure 8: FT-IR Spectrum of Reference Standard Glutathione

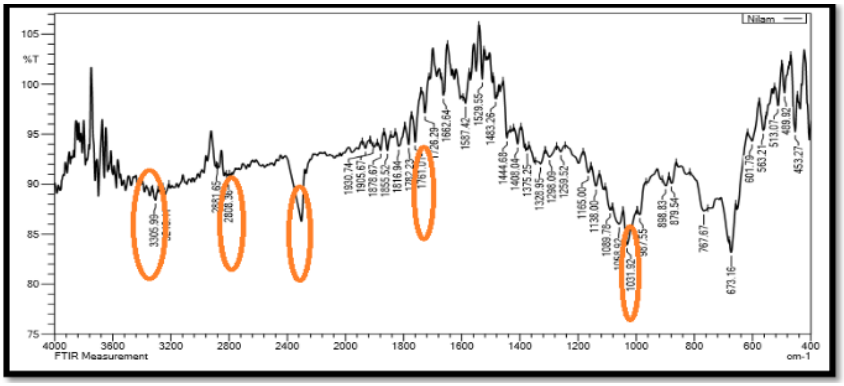

Figure 9: FTIR of Physical Mixture of Drug and all the Excipient (Sodium bicarbonate and Trehalose)

Table 15: Comparison of FTIR Spectrum of drug sample and reference standard

Sr. No.	Functional Groups	Peaks of Functional Groups (cm^{-1})	
		Reference Standard	Physical mixture
1	N-H_2 (1° Amine) Stretch	3340	3305.95
2	O-H	3025	3210.36
3	C=O	1720	1761.01
4	S-H	2550	2400
5	C-N	1020	1031.92

DSC Analysis for determination of glass transition temperature (Tg):

DSC Thermogram of formulation shown in Figure – 7 indicates that the classical exothermic peak also known as "S curve" resulting from glass transition temperature (Tg) of formulation and which was within the range of -20 ºC to -30 ºC temperature.

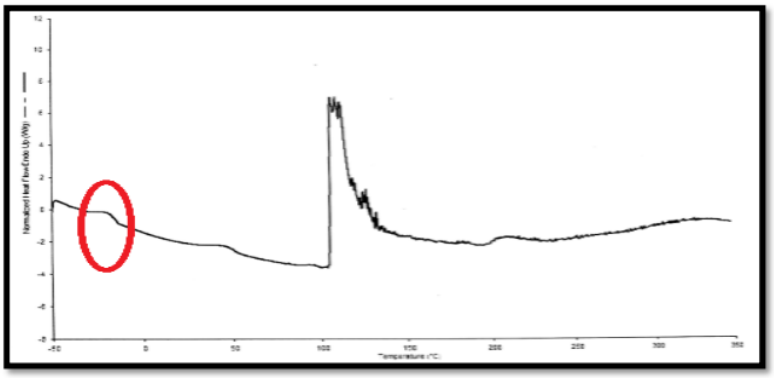

Figure 10: DSC thermogram for glass transition temperature (Tg) of formulation

Preliminary Study

Preliminary study was carried out with the solubility study of drug in water. Then lyophilization cycle was developed on trial and error basis to assess the effect of process parameter as well lyoprotectants on the lyophilized product. The lyophilized cycle was developed with consideration of glass transition temperature,

drying time and vacuum. Here complete collapse of A1 and partial collapse of A2 trial was observed. There for A1 was dropped and next lyophilization cycle was carried out for A2 batch. In which primary drying was started from -35 ºC which affect the appearance of product. Here partial collapse observed in the product. There for the temperature of next stage was set at 15 ºC to observe the appearance of lyophilized product. The obtained product get melt back in this cycle therefore in the next cycle primary drying is divided into four stages where drying was done and product was obtained with shrinkage at bottom. To overcome the problem of shrinkage the next cycle is carried by taking lyoprotectants such as mannitol, lactose, trehalose and sucrose in 5% and 10% concentration. The 5% trehalose containing formulation had good appearance as compared to other formulation so trehalose in 5% concentration was finalized for further studies. After evaluation of product of L1 trial of cycle 5 the next cycle was develop in consideration of reduce the moisture in final product to reduce the moisture of product the trial was taken by increasing secondary drying time hence the total time of lyophilization cycle also increased. By this cycle the product was obtained had elegant appearance.

Results of preliminary batches:

After the selection of excipients, lyophilization cycle was run and developed the lyophilized formulation with the different excipients and observed which formulation formed as elegant cake. After running the lyophilization cycle with different excipient it was concluded that the elegant and good cake of trial batch L1 developed by Freeze Drying cycle number 6 (FD6) i.e. glutathione formulation with 5% trehalose was formed with pH 4.95, residual moisture 1.06%, percentage

transmittance 98.80%, assay 100.5% and reconstitution time 35 seconds as compared to other excipients, which complies with the limits.

Table 16: Results of preliminary batches for development of lyophilization cycle

FD cycle	Trial batch	Evaluation parameter					
		Physical characteristics	pH	Residual moisture (%)	% Transmittance	Assay (%)	Reconstitution time (sec)
FD1	A1	Complete collapse of cake	-	-	-	-	-
	A2	Partial collapse of cake	4.89	7.70	99.54	97.9	50
FD2	A2	Partial collapse and cracks on surface	4.95	7.51	98.91	96.5	45
FD3		Melt back of cake	-	-	-	-	-
FD4		Shrinkage of cake	4.84	6.19	98.40	98.7	65
FD5	M1	good cake but slight shrinkage	4.89	7.10	99.63	98.6	90
	M2	Shrinkage at bottom	4.92	7.03	99.75	94.2	95
	L1	Slight collapse	4.95	7.09	99.70	96.8	60
	L2	Slight collapse	4.91	7.07	99.35	94.3	55
	T1	**Good cake**	**4.98**	**7.01**	**99.92**	**96.3**	**45**
	T2	Good cake but slight depressed at top	4.93	7.52	99.79	97.1	60
	S1	Cracks on top surface	4.99	7.71	99.40	95.6	60
	S2	Slight cracks on top surface	4.83	7.23	99.38	97.2	50
FD6	**T1**	**Good cake**	**4.95**	**1.06**	**98.80**	**100.5**	**35**

Optimization of lyophilization cycle by reducing time: (cycle time reduction)

Long lyophilization cycle are expensive and time consuming. There for lyophilization cycle should be optimized in order to reduce the final cost of the formulation and save the time. After formulation development i.e. formation of elegant cake of trial batch L1 developed by FD cycle number 6 (FD6) i.e. glutathione formulation with 5% trehalose considered as optimum formulation and further study was carried out with this formulation. The process was optimized by reducing the primary drying time at first step of primary drying which is responsible for maximum removal of unbound water. Cycle time was reduced till the lyophilized cake remains elegant and having good characteristics and comply with desired product profile.

Table 17: Optimum formulation for optimization of lyophilization cycle.

Ingredients	Quantity
API	600 mg
Sodium bicarbonate	160 mg
Trehalose	30 mg
WFI	Q. S. to 2 ml

Table 18: Desired product profile

Parameter	Range
Moisture content	NMT 3%
Reconstitution time	NMT 1 min
Assay	98% to 101%
pH	4.5 to 5.5

Table 19: Results of optimized batches

FD cycle	Trial batch	Evaluation parameter					
		Physical characteristics	pH	Residual moisture (%)	% Transmittance	Assay (%)	Reconstitution time (sec)
FD7	T1	Melt back of cake	-	-	-	-	-
FD8		Slight collapse	4.89	5.95	99.71	95.1	45
FD9		Good	4.92	1.18	99.54	97.9	35
FD10		Good	4.96	1.19	99.78	96.6	40
FD 11		Good	4.93	1.19	100.1	99.4	30
FD12		Slight collapse	4.89	1.22	99.17	94.9	45

Initially the study was started by reducing time at first step of primary drying by half of total time in FD7 and the melt back of cake was observed. In next cycle i.e. FD8 the time is reduced by 1/3rd of total time and slight collapse was observed in lyophilized cake. In FD9, FD10 and FD11 the time was reduced by 2, 4 and 6 hours from 2880 min and good cake was observed. In FD12 the time was reduced by 8 hours from 2880 min and slight collapsed cake was observed. Therefor it was concluded that if the further study is carried out again by reducing time cake collapse could be occur so no further study was carried out again. As shown in Table 16 it was concluded that, trial batch L1 developed by FD cycle number 11 (FD11) possessed the desired product profile and this cycle consume minimum time so it was considered as optimized batch. Reduction of primary drying time at the initial stage show in Figure 14 for the comparison of time reduction between FD7 to FD12.

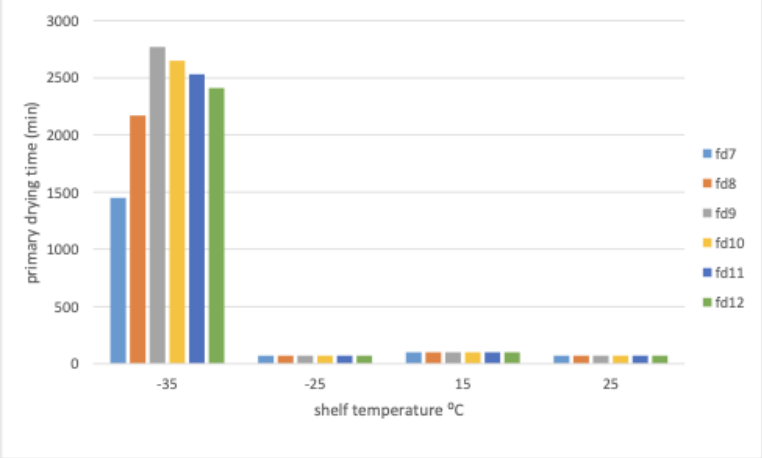

Figure 11: Comparison of Primary Drying Time Vs Shelf Temperature for FD7 to FD12

Characterization of optimized formulation:

X-ray diffraction Analysis:

The X- ray diffraction pattern of pure Glutathione and optimized product was done using scintaz, X-ray powder diffractometry in the range of 20-60º two theta, 0-6000 cps and from the results it was concluded that Glutathione possess crystalline nature and various peaks were shown in Figure – 12. While the optimized formulation was found amorphous in nature as shown in Figure 13. Due to amorphous nature of product it shows minimum reconstitution time i.e. 25 second.

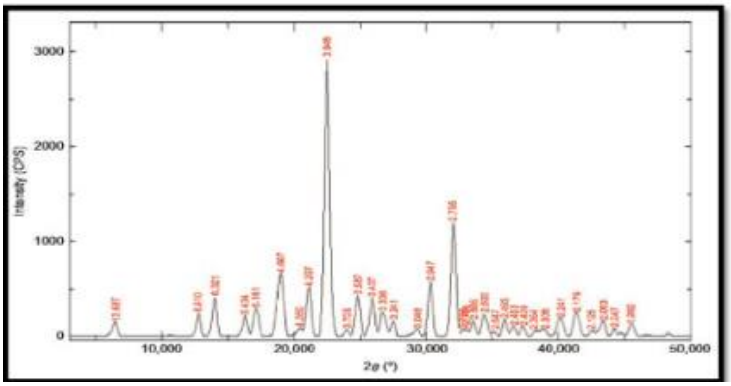

Figure 12: X-Ray Diffraction pattern of pure drug

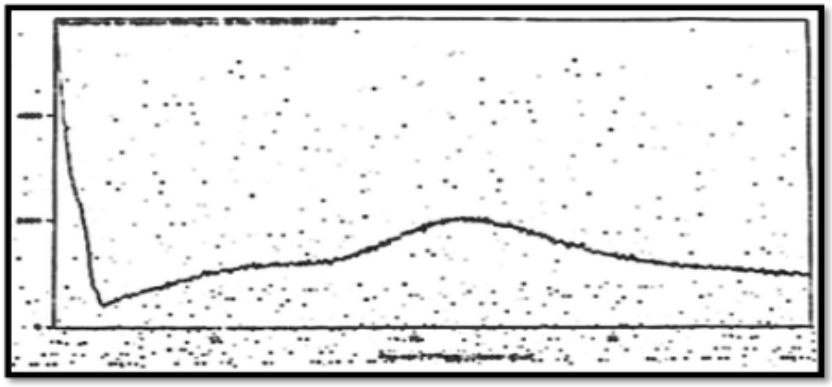

Figure 13: X-Ray Diffraction pattern of optimized product

DSC Analysis:

From the study of DSC thermogram of pure drug shown in figure - 14 the sharp peak was observed that indicates that drug possess crystalline nature. And from the DSC thermogram of pure drug shown in Figure – 15 the broad and less intense peak was observed that indicates that the optimized formulation possess amorphous nature.

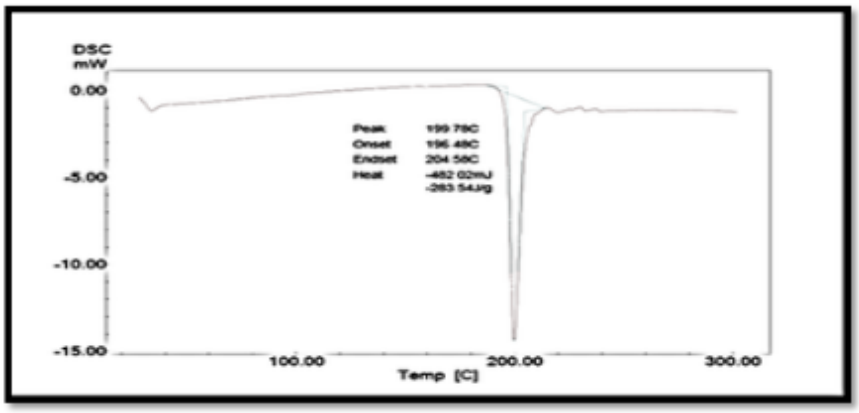

Figure 14: DSC Thermogram of pure drug

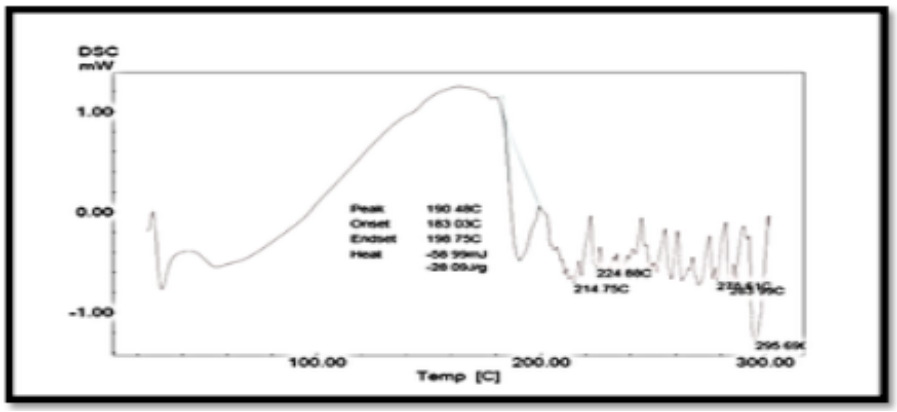

Figure 15: DSC Thermogram of optimized formulation

(a) (b) (c) (d) (f)

Figure 16: Lyophilized formulation (a) cracks on surface of product, (b) complete collapse of product, (c) melt back of product, (d) partial collapse of product, (e) good cake.

Results for reconstitutional stability of optimized formulation:

After the process optimization the final formulation was subjected to reconstitution stability. The reconstitution stability of the formulation was evaluated upto 8 hours with the interval of one hour. From the Table 20 it was concluded that the reconstituted optimized formulation was stable upto 8 hours.

Table 20: Result for reconstitutional stability of optimized formulation

		Time for 8 hours							
		After 1 hours	**After 2 hours**	**After 3 hours**	**After 4 hours**	**After 5 hours**	**After 6 hours**	**After 7 hours**	**After 8 hours**
Final product	Reconstitution stability	Clear and stable	Clear and stable	Clear and stable	Clear and stable	Clear and stable	Clear and stable	Clear and stable	Clear and stable

Results of short term accelerated stability study:

Optimized formulation containing vials were subjected for stability at 40 ± 2°C and at 75 ± 5% RH and after 30 days, the sample were taken and their physical characteristics, pH, residual moisture, percentage transmittance, assay and reconstitution time were checked. After stability period, shows that there was no significant change in the characteristics of optimized formulation during 30 days.

Table 21: Results of accelerated stability study at (40°C/75 % RH) for optimized formulation.

Evaluation Parameters	**Results of optimized batch**		
	Before 30 days	**After 30 days**	**% Bias**
Physical characteristics	Good cake	Good cake	---
pH	4.93	4.95	-0.40
% Residual Moisture	1.19	1.21	-1.65
% Transmittance	100.1	99.89	0.21
Assay (%)	99.4	99.1	0.3
Reconstitution time	30	30	0

CONCLUSION

The present research work was design to develop a lyophilized injectable dosage form of Glutathione lyophilized injection was successfully developed by lyophilization method. The drug is soluble in water but to achieve desire pH and solubility profile of drug sodium bicarbonate is used. The Glutathione is unstable if dispensed as liquid dosage form. Hence the present project was envisage to overcome the drawbacks associated with Glutathione and to formulate a stable formulation by lyophilization technique.

Then glutathione injection was formulated and lyophilized by varying the lyophilization cycle parameters. But, when glutathione was lyophilized alone the cake collapse was observed. Hence, in order to overcome this problem lyoprotectants (mannitol, lactose, trehalose and sucrose) were added

in different concentration i.e. 5% and 10%. The lyophilization cycle was developed for these formulations by changing the process parameter as per the preliminary batches. After formulation development, lyophilization cycle was optimized by reducing the total cycle time. Cycle time reduced at the initial stage of primary drying till the formulation remained stable. First stage of primary drying was reduced to 2520 minutes from 2880 minutes where the cake remains elegant.

The batch L1 developed by FD cycle 11 (FD11) with 5% trehalose having total lyophilization cycle time 59.75 hours was considered as optimized formulation because it exhibited a good cake formation and pH, moisture content, reconstitution time and assay was found within the range of desired product profile. Short term stability studies were conducted for the optimized formulation as per ICH guidelines for a period of one month which revealed that the formulation does not show any significant change in product. From the above results, it can be conclude that the lyophilization technique proves to be an advantage for development of stable injectable dosage form of Glutathione, hence our objective to develop a stable and effective lyophilized injection of Glutathione was achieved.

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