Research Article

FORMULATION AND EVALUATION OF ZOLEDRONIC ACID FOR INJECTION BY LYOPHILIZATION TECHNIQUE

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ABSTRACT

The development of lyophilized injection of zoledronic acid, mannitol used as lyophilisation aid, sodium citrate as buffering agent and water for injection as solvent. The filled vials were loaded into lyophilizer and lyophilized them as per standard cycle and for development of stability for reconstitution. The objective of the project is to formulate the reconstitution solvent for lyophilized zoledronic acid injection for same solvent used for intravenous bolus as well as intravenous infusion administration and better stability of formulation. Quality control tests like description, pH, related substances, moisture content, reconstitution time, assay and particle count were checked according to Pharmacopoeial method and present within the specified limits. The lyophilized product of all the formulations (F1 - F6) prepared were an appearance of white to white crystalline powder. The pH of all the formulations is in the range of 3.5-6.99. These pH values of all the formulation are within the limit. The related substances in formulations not exceeded the limit of 0.5%. The assay values of formulations (F2-F6) were in the range of 93.6 % – 101.43 %. The formulations (F2- F6) show a water content range of 0.78 to 2.39 %. The formulation F4 was one of the best optimized compositions; it was used for reconstitution stability. The results concluded that the formulation F4 is the optimized and the best formulation.

Keywords: Lyophilization, Reconstitution time, Sodium citrate, Zoledronic acid.

INTRODUCTION

Parenteral products are intended for administration by injection injectables (i.e., solutions, suspensions, emulsion and dry powers for reconstitution), intramammary infusions, intravaginal delivery systems or implantation through the skin, or other external layers such as stratum corneum and directly into body fluids, tissues, or organs. The medicament is readily transported from the site of administration to its site of action.

Lyophilization or freeze drying is a process in which water is removed from a product after it is frozen and placed under a vacuum, allowing the ice to change directly from solid to vapor without passing through a liquid phase ^{1,2}. Freeze drying also known as lyophilization, is widely used for pharmaceuticals to improve the stability and long term storage of labile drugs³.

Lyophilization is used for stabilization of various pharmaceutical products, including virus vaccines, protein and peptide formulations, and liposome and small- chemical drugs^{4,5}.

Zoledronic acid⁶ is a highly potent third generation bisphosphonates. It is used for treatment of hyper glycaemia, bony metastasis and Paget's disease.

Chemical structure of zoledronic acid

Most of the drugs are unstable in solution form like cisplatin, bendamustine hydrochloride. It will degrade within hours when it was in solution form. The powder form is having the advantage of decreased moisture content and hence increases the stability of the product. An attempt was made to formulate and stabilize the zoledronic acid for injection by lyophilization or freeze drying process.

MATERIALS AND METHODS

Materials: Zoledronic acid procured from Natco Pharma Ltd, Hyderabad; Mannitol, Signet chemicals and Sodium citrate, Sheffield Pharma.

Equipment's: Balance, Mettler- Toledo, USA; pH meter, Digisun Electronics, Hyderabad; Magnetic stirrer measuring instruments, Remi equipment, Mumbai, India; Osmometer, Adventure oxas, Germany; Freeze drying equipment, Pamas, Bangalore, India; Measuring cylinder S.S vessel, Merck, Germany; High-pressure liquid chromatography, Waters 2695,

USA; Water Content Karl Fischer, Mettler Toledo DL50 Graphix, USA; UV-Visible, Shimadzu, Malaysia; Particle Count Analyzer, Pamas, Bangalore, India.

Preformulation Studies

Solubility: The solubility of drug is an important physicochemical property because it affects the bioavailability of the drug. Solubility is determined by equilibrium solubility method, which involves stirring the drug in a solvent for a prolonged period of time until equilibrium is achieved.

Melting point: The melting point of the drug sample was determination by capillary method. Drug was taken in the glass capillary whose one end was sealed by flame; the capillary containing drug was dipped in the liquid paraffin inside the melting point apparatus.

Drug-excipient compatibility studies: The proper design and the formulation of dosage form require consideration of

physical, chemical and biological characteristics of the drug and excipients used. From the review it was known that the drug itself is not stable in the solution form hence it is prepared as a lyophilized product. The drug and excipients must be compatible with one another to produce a product that is stable, efficacious, safe and easy to administrate. The compatibility studies are carried out at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \pm 5\% \text{ RH}$ (for 2 weeks) and $40^{\circ}\text{C} \pm 2^{\circ}\text{C}/75\% \pm 5\% \text{ RH}$ (4 weeks).

Compatibility studies

Preformulation studies were carried out to study the compatibility of the pure drug zoledronic acid with mannitol and sodium citrate.

IR spectra of the pure drug and excipients were obtained which is shown in figures 1 to 4 and table 1. The principal peaks obtained for the combinations were almost similar to that of the drug, there was no significant difference in the IR spectra of pure zoledronic acid and physical mixtures of excipients of drug.

Table 1: IR spectra data of zoledronic acid

Frequency (cm^{-1})	Assignments	Frequency (cm^{-1})	Assignments
1642.26	CH=CH Stretching	1193.12	P=O Stretching
1406.52	C-H stretching	1293.62	P=O Stretching
928.41	C-C Stretching	2806.31	-CH ₂ Stretching
1586.02	C-N stretching	3204.74	-OH Stretching
1341.36	Alcohol

Figure 1: FTIR Spectra of the zoledronic acid

Figure 2: FTIR Spectra of the sodium citrate

Figure 3: FTIR Spectra of Mannitol

Figure 4: FTIR Spectra of Zoledronic acid + Sodium citrate + Mannitol

Table 2: Composition of Formulations (F1-F6)

S.No	Ingredient	F1	F2	F3	F4	F5	F6
1	Zoledronic acid(mg/mL)	2	2	2	2	2	2
2	Mannitol (mg)	90	95	100	110	115	120
3	Sodium citrate (mg)	6	8	10	12	13	14
4	Water For Injection(mL)	qs to 1 mL	qs to 1 mL	qs to 1 mL	qs to 1 mL	qs to 1 mL	qs to 1 mL

Development of method for Zoledronic acid for injection

In formulation F1, the water for injection content in the formulation is more; the moisture content in the lyophilized product is more. The pH of the drug is less (acidic). So the solubility of the drug is low and the assay of the finished product is less, there by the reconstitution time is more. Hence, author made an attempt for another formulation with increasing the concentration of both mannitol and sodium citrate in the formula.

In formulation F2, increased concentrations of mannitol (95mg) and sodium citrate(8mg) is used. So the moisture content in the formulation is decreased than the F1 and the pH of the solution is increased. So the solubility of the drug is also increased there by increasing the assay compared to F1. But the reconstitution time is still more. The residual solvents ppm is increased in F2 compared to F1 because as there is increase in the concentration of mannitol and sodium citrate. So the next formulation is planned with further increase in concentration of mannitol and sodium citrate.

Formulation F3, the pH of the solution is increased so the solubility of drug is more compared to the solubility in F2. So there is an increase in the assay of formulation compared to F1 and F2. And as the solubility increases the reconstitution time decreases. The percentage of water absorbed is more. So the water content in the final product was also low. To get even better results trials are planned with the increase in concentrations of mannitol and sodium citrate.

In formulation F4, the drug zoledronic acid gets completely solubilised in the water for injection leaving a clear solution as the pH of the solution is within the limits. So there is 100% assay for the final lyophilized product. The reconstitution time is decreased to less than one minute. For a lyophilized powder reconstitution time, water content, and related substances plays critical role. Increase the concentration of mannitol results in gradual increase in assay of the formulation. It may be because of minimizing the hydrolysis of drug by decreasing the water percentage in the formulation. To reconfirming the above statement, further formulation is planned with increasing the concentrations of mannitol and sodium citrate in the formulation.

In formulation F5, the drug zoledronic acid is completely solubilised leaving a clear solution, but the pH of solution was found to more. The assay is increased because of decrease in hydrolysis as the mannitol concentration has increased in the formulation. The reconstitution time is also decreased compared to previous formulations. The percentage of water content of the final product has also decreased because of increase in the concentration of mannitol and decrease in the percentage of water as it is been absorbed. So the cake of the drug was found to be hard.

As the percentage of sodium citrate increases the related substances of the final product also increases, this is reached almost the specification limit of the zoledronic acid formulation which is not preferred.

In formulation F6, the drug zoledronic acid is completely solubilized leaving a clear solution. This has resulted in an increase in the assay of the formulation. But the pH was found to be very high. The water content of the lyophilized vials is also decreased because of increased concentration of mannitol. So the formed cake was too hard and there is an increased reconstitution time. But the pH of the final product crossed the specified limit of the zoledronic acid formulation which is found to be toxic. So this concentration of sodium citrate is not recommended for the preparation of the zoledronic acid formulation. And the lyophilized cake of the drug was found to be very hard, so this concentration of mannitol is not recommended for the preparation of zoledronic acid for injection.

Apart from the last two formulations, the formulation F4 has the optimized concentration of mannitol and sodium citrate which is good for the formulation of the product with best results.

Description

The lyophilized product of all the formulations (F1-F6) prepared was subjected for visual inspection where all the products are giving an appearance of white to white crystalline powder. The results are shown in the table 6.

Ph

The pH of all solutions was determined by pH meter. The pH was varied with change the concentration of the sodium citrate. The pH of all the formulations is ranging from 3.5-6.99. These pH values of all the formulation are within the limit. All the formulations pass the pH test.

Related Substances

The related substances present in the formulations F1-F6 are determined using the HPLC method. The total value of the related substances should be not more than 0.5%. The formulations not exceeded the limit of 0.5%. So all the formulations are within the limits of the prescribed % of related substances and pass the test.

Assay

The formulation F5 had shown the highest value of 104.29 %. The formulation F6 has shown 103.75 %. The remaining formulations have given an assay values ranging from 93.6 % – 101.43 %. The assay value can range from 95.0%-110.0% as per the limits. So except the formulation F1 all the formulations stand with in the specifications in case of assay. Formulation F1 doesn't stand with in the specified assay range.

Water Content

The parameter checked for the drug in the formulation. The zoledronic acid is formulated as a lyophilized product; it is not stable in the solution form. The water content is checked by Karl Fisher titration method. The water content should NMT 2.5% w/w. The formulation F1 shows water content of about

3.17%, this above the specified limit, so the formulation F1 doesn't pass the water content test. The remaining formulations F 2- F6 shows a water content range of 0.78 to 2.39 %, which are within the limit so all the formulations except F1 pass the water content test.

Reconstitution Time

The time taken for reconstituting the lyophilized cake with the 0.9% NaCl solution is checked for all the batches and the time is noted. The reconstitution time should be less than 2 minutes. Except the formulation F1 all the other formulations F2, F3, F4, F5 and F6 passes the test. Hence, the reconstitution time for the formulation F1 is 2 min and 50 sec, it doesn't pass the test.

Sub visible particles

The sub visible particles are separated into 2 parts, in which particles with size $\geq 10 \mu$ and $\geq 25 \mu$, the limits are 6000/vial and 600/vial respectively. In all the formulations the sub visible particles are within the limits so all the formulations pass the test.

Sterilization

Dry heat sterilization and depyrogenation are pure heat treatment, suitable for glassware and metal as well as liquids with low moisture content. This method is also very suitable for heat treatment of powder medicaments. Dry heat sterilization and depyrogenation is a complete destruction of micro-organisms by means of dry heat for a controlled period of time.

Sterilization of vials

Hot-air oven

Dry heat is less effective than steam for sanitizing and sterilizing, but many brewers use it. Temperature about 100 °C is maintained for about 20 min for sterilization.

Sterilization of Rubber Stoppers

Steam at atmospheric pressure

Moist heat (steam under pressure) in the form of saturated steam under pressure is the most widely used and the most dependable method. The recommendations for sterilization in an autoclave are 15 minutes at 121°C (200 kPa). The temperature should be used to control and monitor the process; the pressure is mainly used to obtain the required steam temperature.

Manufacturing Procedure

Raw material verification

Take 10 lit of freshly collected WFI in a 25 lit pressure vessel and allow it to cool at 30 to 50°C. Take 8.5 lit of water for injection in step 1 in 10 lit pressure vessel and keep nitrogen purging for 10 minutes. Check and weight zoledronic acid and add step 2 with continuous stirring. Check and add mannitol to step 3 with continuous stirring. Check clarity of the solution for homogenous mixing. Make up the volume to 10 lit with WFI. Adjust the volume of step 4 on Wt/ml basis up to 10.20 kg.

In process check

Withdraw 25 ml sample for analysis as per current approved in process specifications. (a). Assay: 1.96-2.04mg/ml (b). Weight/ml – 1.04g/ml. Check and weight sodium citrate and dissolve in step 5 bulk solution for homogeneous mixing. Check the pH of the solution. It should be between 5.7 - 6.7. Record the pH.

Bulk In Process Check

Withdraw sample for analysis as per current approved in process specifications (pH: 5.7-6.7). Filtration of bulk solution in aseptic condition should be done before filtration. Filter integrity test should be performed. (a). Pre integrity test (b). Post integrity test

Filling of vials

1.98 ml solution is filled in to 6ml tubular vials (20mm neck), the vials are half stoppered with 20 mm lyophilized stoppers. The filled vials are loaded into a lyophilizer and the vials are lyophilized as per cycle (Set the shelf temperature 5°C, prior to cycle start). The samples are collected at the end of lyophilization cycle. The samples are withdrawn under the nitrogen atmospheric conditions after the atmospheric pressure is reached. These vials are stoppered and sealed. The vials are unloaded from the lyophilizer and sealed by using flip off aluminum seals

The vials are stored at room temperature.

RESULTS AND DISCUSSION

Solubility Determination

Water solubility of zoledronic acid

Add approximately 1 ml of water to the test tube containing zoledronic compound. Shake the tube and/or stir with a glass stirring rod. An insoluble zoledronic acid is appeared, increasing the volume of water up to 20 ml it is insoluble, but it is soluble sparingly in 0.1N sodium hydroxide. The hydrate form of the zoledronic acid mono hydrate is freely soluble in water.

Table 3: Solubility study of zoledronic acid monohydrate

Zoledronic acid	Solubility
1mg/ml	Freely soluble
5mg/ml	Freely soluble
10mg/ml	Freely soluble
15mg/ml	Freely soluble
20mg/ml	Sparingly soluble

Zoledronic acid monohydrate was freely soluble up to 15mg/ml. After this increasing the amount of drug and decreasing in the solubility.

Solution Stability Studies

The pH values and assay of the solution are near to the standard values. Bulk solution stability checked by keeping at normal room temperature (25°C) with different concentrations of sodium citrate. The buffer quantity may affect the clarity and pH of the solution

Table 4: Zoledronic acid solution stability study with sodium citrate

S. NO	Parameter	Solution stability with 8mg sodium citrate						Solution stability with 10mg sodium citrate					
		Initial	3 h	6 h	9 h	12 h	24 h	Initial	3 h	6 h	9 h	12 h	24 h
1	Description	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
2	pH	7.5	7.8	7.6	7.5	7.8	7.6	7.0	7.2	6.9	6.7	6.9	6.9
3	Assay (%)	101	97.4	94.2	92.8	88.7	84.2	100.4	99.5	98.4	95.7	91.1	84.9
4	Related substance												
	Single Max (%)	1.07	3.01	5.67	6.08	6.54	10.32	1.37	3.42	6.71	7.95	9.38	12.49
	Total Imp (%)	1.98	5.96	8.12	8.58	12.01	16.6	1.51	4.93	8.70	9.34	12.10	17.06

Above results showed that the pH is very high, increasing the time duration the thermal degradation shows more significant degradation of assay and also drastic increase in impurities also observed based on above results further studies was planned to conducted at 2-8°C. Above thermal degradation due to heat sensitive of drug to prove that further trials was conducted at 2-8°C.

hold time in different concentration of sodium citrate. Significant decrease of assay and increase in related substances was observed when increasing the hold time at 25 °C.

Zoledronic acid formulation is unstable in solution form in all the concentrations of sodium citrate when stored at 25°C. Increasing the concentration of zoledronic acid decreasing the degradation is observed.

The test samples are clear, colorless solutions at the end of study. The pH of the samples decreased when increasing the

Table 5: Zoledronic acid solution stability study with sodium citrate

S. NO	Parameter	Solution stability with 11mg sodium citrate						Solution stability with 12mg sodium citrate					
		Initial	3 h	6 h	9 h	12 h	24 h	Initial	3 h	6 h	9 h	12 h	24 h
1	Description	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
2	pH	6.7	6.8	6.9	6.8	6.7	6.8	6.34	6.33	6.32	6.3	6.3	6.3
3	Assay (%)	104	102.2	99.98	99.5	98.4	95.7	100.5	100.2	100.6	101.0	100.5	100.0
4	Related substance												
	Single Max (%)	0.93	2.47	3.15	3.87	4.66	6.86	0.106	0.073	0.156	0.221	0.272	0.685
	Total Imp (%)	1.05	2.71	3.48	4.29	5.17	7.73	0.237	0.242	0.506	0.806	0.987	1.515

Table 6: Post Formulation Evaluation Parameters

Parameters	F1	F2	F3	F4	F5	F6
Description	***	***	***	***	***	***
pH	3.5	4.65	5.3	6.15	6.55	6.99
Related substances	0.32%	0.40%	0.29%	0.15%	0.50%	0.48%
Assay	93.6%	97.8%	99.2%	102.43%	104.29%	103.75%
Water content	1.631%	1.201%	1.199%	0.937%	0.932%	0.89%
Reconstitution time	2min 50sec	1min 40sec	1min 13sec	55sec	43sec	45sec
Sub-visible particles						
>=10micron	1413	3637	2604	781	1195	4252
>=25micron	19.8	31.8	75	10.8	24	72

***White to white crystalline powder

Table 7: Estimation of zoledronic acid assay by HPLC method

Parameters	Innovator	F1	F2	F3	F4	F5	F6
Description	***	***	***	***	***	***	***
pH	6.01	3.5	4.65	5.3	6.15	6.55	6.99
Related Substances	0.48%	0.32%	0.40%	0.29%	0.15%	0.55%	0.48%
Assay	101.6%	93.6%	97.8%	99.2%	101.43%	104.29%	103.75%
Water content	0.89%	1.631%	1.201%	1.199%	0.937%	0.932%	0.89%
Reconstitution Time	58 sec	2min 50sec	1min 40sec	1min 13sec	55sec	43sec	32sec
Sub-visible particles							
>=10micron	3595	1413	3637	2604	781	1195	4252
>=25micron	91.8	19.8	31.8	75	10.8	24	72

*** White to white crystalline powder

Drug excipient compatibility studies

FTIR spectra of zoledronic acid appears to be as same as the FTIR spectrum of physical mixture at same wave number indicating no modification or interaction between the drug and additives indicated that they were compatible with each other and the results are shown in table 1.

Sterilization: Sterilization of vials by using hot-air oven and the rubber stoppers are sterilised by using moist heat sterilization involves the use of steam in the range of 121-134°C.

Manufacturing procedure: Manufacturing process involves collection of raw materials, and its verification of weights and then mixing of the ingredients according to Batch manufacturing record.

In process check: In process check was done for its assay and weight/ml.

Bulk in process check: The pH of the solution was measured and the solution was filtered aseptically. The pH was of solution was in the range of 4.5 to 7.

Particulate matter: It can be done by visual inspection. It is essentially free from particles of foreign matter that can be seen on visual inspection. There are no visible particles observed in the solution.

Assay: The drug content of the solution was found between 95.0 to 110.0%.

Related substances: The related substances are found in the solution and it will be in the specified limits.

Imidazole	Not more than 0.5%
1-Imidazole acetic acid	Not more than 0.5%
Individual Unknown impurity	Not more than 0.1%
Total impurities	Not more than 2.0%

Sub visible particles: The sub visible particles found and determined by using sub visible particle counter. The values are within the limits.

$\geq 10\mu\text{m}$	Not more than 6000/vail
$\geq 25\mu\text{m}$	Not more than 6000/vail

Moisture content: Take 20 ml of methanol into the titration vessel and titrate the amperometric end point with karlfischer reagent. Quickly add 0.1 gm of the sample, accurately weighed, stir for 1 minute and again titrate to the amperometric end point with karlfischer reagent. Perform duplicate. It is not more than 2.5% w/w.

The results of all the six formulations were tabulated and concluded that the formulation F4 was the optimized formulation with the best evaluation parameters.

Table 8: Comparative analytical studies on reference listed drug (ZOLDONAT) and zoledronic acid for injection F4

Evaluation parameters	Reference drug	Formulation F4
Description	White to white crystalline powder	White to white crystalline powder
pH	6.01	6.15
Related Substances	0.48%	0.15%
Assay	101.6%	102.43%
Water Content	0.89%	0.937%
Reconstitution Time	58 sec	55sec
Sub-visible particles		
$\geq 10\mu\text{m}$	3595	781
$\geq 25\mu\text{m}$	91.8	10.8

The above table gives the comparative results of the evaluation parameters of the RLD (Zoldonat) which is manufactured using the lyophilization cycle and the formulation F4 which is done using lyophilization cycle period.

From the results it is concluded that the results of the RLD are less efficient than the evaluation results of the formulation F4 especially in the particle count test.

Accelerated Stability Studies

From the above accelerated stability study results, it was observed that the storage of the formulation at elevated temperatures for a period of 1st month lead to the degradation of the product resulted in decrease in assay and increase in the reconstitution. After the end of the period of 2nd and 3rd the change was even more drastic. So it was understood that the zoledronic acid for injection formulation should be stored only at room temperature 25°C but should not be exposed to elevated temperature.

**Table 9: Accelerated stability studies for formulation F4
(Condition: 40°C $\pm$ 2°C/75% RH $\pm$ 5% RH)**

Test	1 month	2 month	3 month
Description	White to white crystalline powder	White to white crystalline powder	White to white crystalline powder
pH	5.98	5.98	5.96
Related substances	Nil	Nil	Nil
Assay	103.3%	103.0%	102.0%
Water content	0.66%	1.10%	1.17%
Reconstitution time	60sec	1 min 20sec	2min 10sec

SUMMARY AND CONCLUSION

Zoledronic acid is a bisphosphonate used for the treatment of osteoporosis, which is not soluble in the water and it is unstable in solution form, hence zoledronic acid was formulated in the form of lyophilized dry product for injection.

The results concluded that the formulation F4 is the optimized and the best formulation. The evaluation parameters like assay, reconstitution time, related substances, particulate matter and water content for the formulation F4 are within the specified limits.

The comparative results between the marketed product (ZOLDONAT) and zoledronic acid (formulation F4) have shown that the marketed product (ZOLDONAT) which are manufactured using lyophilization cycle results are comparatively less in efficiency than the zoledronic acid for injection (formulation F4) which was carried out using lyophilization cycle period.

From the accelerated stability studies it is concluded that the zoledronic for injection formulation should be stored at room temperature (25°C) and should not be subjected to high temperatures.

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