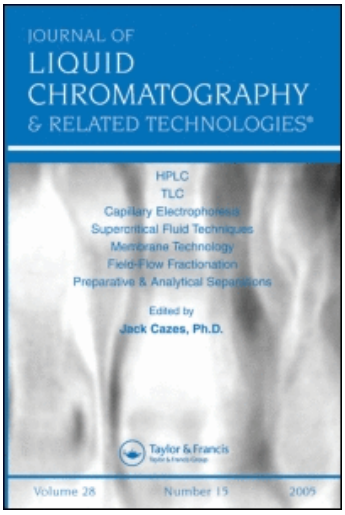

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Regulatory Requirement - Validated, Specific, and Stability Indicating Analytical Method for Zoledronic Acid and Its Related Impurities by Ion Pair Reversed Phase Liquid Chromatography (IP-RPLC)

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Regulatory Requirement – Validated, Specific, and Stability Indicating Analytical Method for Zoledronic Acid and Its Related Impurities by Ion Pair Reversed Phase Liquid Chromatography (IP-RPLC)

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Abstract: A. sensitive and precise ion pair reversed phase liquid chromatographic (IP-HPLC) method was developed and validated for the determination of zoledronic acid in the presence of its impurities and degradants in parental sterile dosage form. The chromatograph employs octadecyl silane chemically bonded to porous silica, stainless steel analytical column with mobile phase of methanol, and buffer at column flow rate of 0.8 mL min^{-1}, temperature thermo stated to 50°C, and a UV-vis detector wavelength of 215 nm. The buffer used in the mobile phase contains phosphate buffer and tetra butyl ammonium hydrogen sulphate as ion pair, and the pH of the buffer adjusted to 7.2 with dilute orthophosphoric acid. The proposed method was established to have sufficient intermediate precision as a similar separation was achieved on another instrument handled by a different operator, and the method was validated for precision, specificity, linearity, range, accuracy, LOQ, ruggedness, and robustness. The method was also successful in the analysis of the drug in marketed samples subjected to stability testing under stressed conditions of pH hydrolysis, oxidation, and temperature stress as per ICH guidelines. Statistical analysis of the data proved that the method is precise and accurate and can be applied for quantification of impurities and actives in pharmaceutical dosage formulation.

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Keywords: Forced decomposition studies, ICH, Stability, Validation

INTRODUCTION

Pharmaceutical product quality is of vital importance for patient safety. The presence of impurities may influence the efficacy and safety of pharmaceuticals. Impurities and potential degradation products can cause changing of chemical, pharmacological, and toxicological properties of drugs having significant impact on product quality and safety. Hence, drug stability is considered to be the secure way to ensure delivery of therapeutic values to patients.[1]

Due to their possible composition, pharmaceuticals are especially sensitive to environmental factors. Strict storage conditions are necessary for the maintenance of integrity and product activity.

Stability is defined as the capacity of a drug substance or drug product to remain within the established specifications in order to maintain its identity, strength, quality, and purity throughout the retest of expiration dating periods. Stability testing of active substances of a finished product provides evidence on how the quality of a drug substance or drug product varies with time, influenced by a variety of environmental factors such as temperature, humidity, and light. The results of stability are applied in the developing of robust manufacturing processes, selecting proper packaging, storage conditions, product's shelf life, and expiration dates.[2–5]

Because the distribution of the environment is highly variable, products must be distributed in a manner that ensures that the product quality will not be adversely affected. The effect of possible temperature and humidity fluctuations, outside of labeled storage conditions, during transportation or storage of drug products, can be evaluated on the basis of the stability analysis for that drug product. Therapeutic application of many drugs raise the need in giving the impurity profile, minor and major degradants and amounts, to ensure the safety of administration as well as concentration levels.

With the advent of the International Conference on Harmonization (ICH) guidelines, the requirement of establishment of a stability indicating method has become more clearly mandated. The guidelines explicitly require conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, etc.; separation of drug from degradation products and the method is expected to allow analysis of individual degradation products.

Accordingly, the purpose of this report is to suggest a systematic approach for the development and validation of the method that should meet the current ICH and regulatory requirements. The discussion also touches upon various critical issues, such as the extent of separation of degradation products, establishment of mass balance, etc., which are

important with respect to the development of stability indicating assays, but are not yet fully resolved.

A review of literature revealed two chromatographic methods[6,7] employed for zoledronic acid. However, the reported method[6] falls short in meeting the current regulatory requirements for impurities quantification as per ICH guidelines for reporting and identification thresholds.[8] Apart from this, there is no sufficient validation and statistical approach for impurities even though the reported paper claims related substances. When the same method was used in our laboratory we found the methods inefficient for separating all the impurities, degradants, and unable to achieve the LOQ for all impurities as per ICH. Hence, the present paper describes the method in detail, such that sufficient information is available for the field chemist to assess the quality of the method. The present research work on the method has an added advantage,[6] in terms of good sensitivity, better retention times for good baseline separations from stress studies, and meets the ICH reporting thresholds requirements for a low dose parental drug product, with wide range of linearity and recovery studies. In the present proposed method, LOQ for Zoledronic acid and imidazol-1-yl-acetic acid are 119 times and 6.5 times more sensitive than the reported method.[6] The results are tabulated in Table 1.

Table 1. Comparisons of results of analytical methods

Parameter	Present method	Method[6]
For Assay		
i. Linearity Range	40–600 $\mu g\,mL^{-1}$	79.96–799.6 $\mu g\,mL^{-1}$
ii. Correlation coefficient	0.9999 (n = 7)	0.9996 (n = 7)
iii. Regression equation	$y = 9539x - 41760$	$A = 7.22 \times 10^3 + 2.82 \times 10^3 C$
For impurities		
i. Linearity Range	LOQ to 4 $\mu g\,mL^{-1}$	Data not available
ii. Correlation coefficient		
Imidazol	0.9999 (n = 7)	Data not available
Imidazol-1-yl-acetic acid	0.9991 (n = 7)	Data not available
iii. Regression equation		
Imidazol	$y = 16506x - 80.93$	Data not available
Imidazol-1-yl-acetic acid	$y = 10913x + 2171$	Data not available
iv. Recovery studies range		
For Assay	200–600 $\mu g\,mL^{-1}$	320.2–480.4 $\mu g\,mL^{-1}$
For Impurities	0.3–2.0 $\mu g\,mL^{-1}$	Data not available
v. LOQ		
(Based on Signal-to-noise ratio)	10:1	20:1
Zoledronic acid LOQ	0.32 $\mu g\,mL^{-1}$	38 $\mu g\,mL^{-1}$
Imidazol-1-yl-acetic acid LOQ	0.17 $\mu g\,mL^{-1}$	1.1 $\mu g\,mL^{-1}$
Imidazol LOQ	0.11 $\mu g\,mL^{-1}$	Data not avilabale

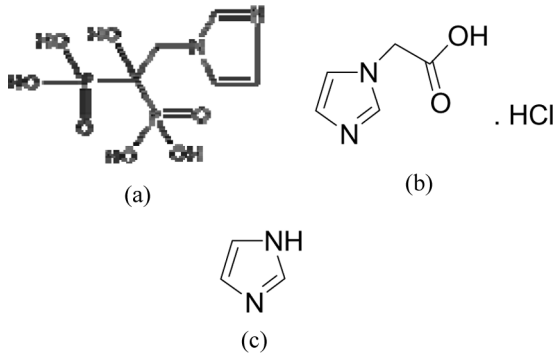

Figure 1. Chemical structures of (a) Zoledronic acid, (b) Imidazol-1-yl-acetic acid and (c) Imidazol.

The method[7] was employed for the analysis of zoledronic acid in bulk samples. Due to lack of specificity for impurities and huge placebo interferences present in the injection dosage form, the above method was not used for zoledronic acid injection. Also a number of reports[9–11] exists on procedures for its determination from biological fluids, such as plasma and urine, using different detection techniques.

Zoledronic acid (1-Hydroxy-2-imidazol-1-ylphosphonoethyl) phosphoric acid (Figure 1) is a bisphosphonte, used to prevent skeletal fractures in patients with cancers such as multiple myeloma and prostate cancer. It can also be used to treat hypocalcaemia of malignancy and can be helpful for treating pain from bone metastases.[12]

EXPERIMENTAL

Reagents and Chemicals

The pure zoledronic acid API, imidazol and imidazole-1-yl-acetic acid impurities used for the development were kindly supplied by Process Research Department of Dr. Reddy's Laboratories Limited (Hyderabad, India) and the respective chemical structures are given in Figure 1. Zoledronic acid injection vials and the corresponding placebos were obtained from Formulations Research Department of Dr. Reddys Laboratories; sodium citrate and mannitol were of analytical grade. Dipotassium hydrogen phosphate anhydrous and tetra butyl ammonium hydrogen sulphate was obtained from Merck, orthophosphoric acid and methanol was obtained from Rankem (India). Sodium hydroxide was purchased from Ranbaxy Laboratories and hydrochloric acid was purchased from LOBA Chemie PVT. Ltd (India). Hydrogen peroxide was procured from s.d.

Fine-chem. Ltd (India). Double distilled water was used throughout the experiment, and other chemicals used were of analytical or HPLC grade.

HPLC Instrumentation

The chromatographic separation was performed on Agilent HPLC 1100 series, Agilent Technologies, USA. The HPLC system consisted of an on-line degasser (G1379A), low pressure quaternary system delivery module (G1311A), auto injector and auto sampler (G1313A), column oven (G1316A), UV-visible detector (G1314A). The output signal was monitored and processed using Empower software (Waters) on a Pentium computer (Digital Equipment Co). Robustness and peak purity testing was done on another HPLC system equipped with a separation module (Waters 2695 model) and photo-diode array detector (Waters 2996 model).

Chromatographic Conditions

Mobile phase consisted of buffer and methanol in the ratio of 900:100, v/v. The pH of the buffer was adjusted to 7.2 ± 0.1 with dilute orthophosphoric acid. The buffer used in the mobile phase consisted of 30 mM dipotassium hydrogen phosphate anhydrous and 5 mM tetra butyl ammonium hydrogen sulphate ion pair in double distilled water. The mobile phase was premixed and filtered through a 0.45 µ membrane filter and degassed. Inertsil ODS-3 V, 250×4.6 mm i.d, 5 µm, (GL Sciences Inc, Japan) stainless steel analytical column was used as stationary phase for successful separations and good peak symmetry. The instrumental settings were performed at constant mobile phase flow rate of 0.8 mL min^{-1}, column temperature thermo stated at $50°C \pm 5°C$, and a UV-vis detector wavelength of 215 nm. Chromatograph conditions for both assay and impurities are the same except for a change in the injection volume. The injection volume was 50 µL for impurities, necessary to achieve LOQ for impurities and zoledronic acid as per ICH[10] and 10 µL for assay.

Analytical Procedures

Standard Solution and Sample Solution Preparation

Mobile phase was used as diluent for the standard and sample preparations. For assay, a standard solution of known concentration of about 400 µg mL^{-1} was prepared. For impurities, a known concentration of about 1.2 µg mL^{-1} of imidazol-1-yl-acetic acid impurity and zoledronic

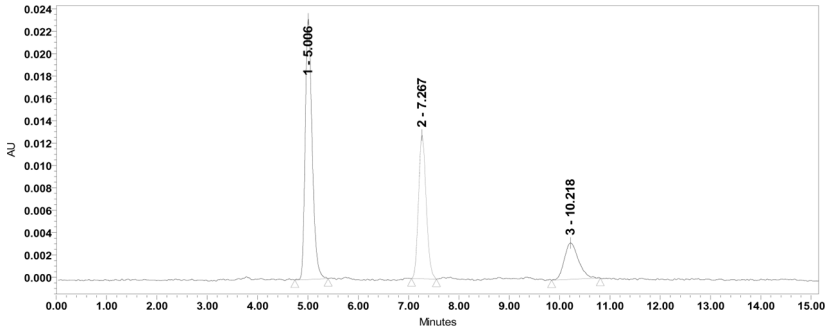

Figure 2. HPLC chromatogram showing system suitability for impurities at 1.2 μg mL^{-1} concentration. Peaks: imidazol impurity, 1; imidazol-1-yl-acetic acid impurity, 2; zoledronic acid, 3.

acid were prepared. The sample preparation is common for both assay and impurities. Each zoledronic acid injection vial contains 4 mg of zoledronic acid as active, 220 mg of mannitol and 24 mg of sodium citrate as excipients. Five sample vials were reconstituted to 50 mL with diluent based on the labeled content of zoledronic acid to achieve a solution concentration of about 400 μg mL^{-1} (Figures 2, 3).

Forced Decomposition Studies for Establishment of Stability Indicating Method

Zoledronic acid injection (drug product) and the placebo solution were used for all decomposition studies. The pH of buffered solutions was measured before and after the reaction and pH was adjusted to near about to 7.0 before sample analysis. All the solutions for use in forced decomposition studies were prepared by dissolving the drug product in small volumes of stressing reagents. After the degradation, these solutions were diluted with diluent to yield a stated concentration of 400 μg mL^{-1}. Conditions employed for performing stress studies were as follows.

Hydrolytic Studies

Acid degradation studies were performed by heating the drug product solution in 1.0 M HCl at 80°C on a water bath for 6 hours. The studies in alkaline conditions were done in: 1.0 M NaOH at 80°C for 6 hours. For the study in neutral conditions, the drug product solution in water was heated at 80°C for 6 hours prior to analysis.

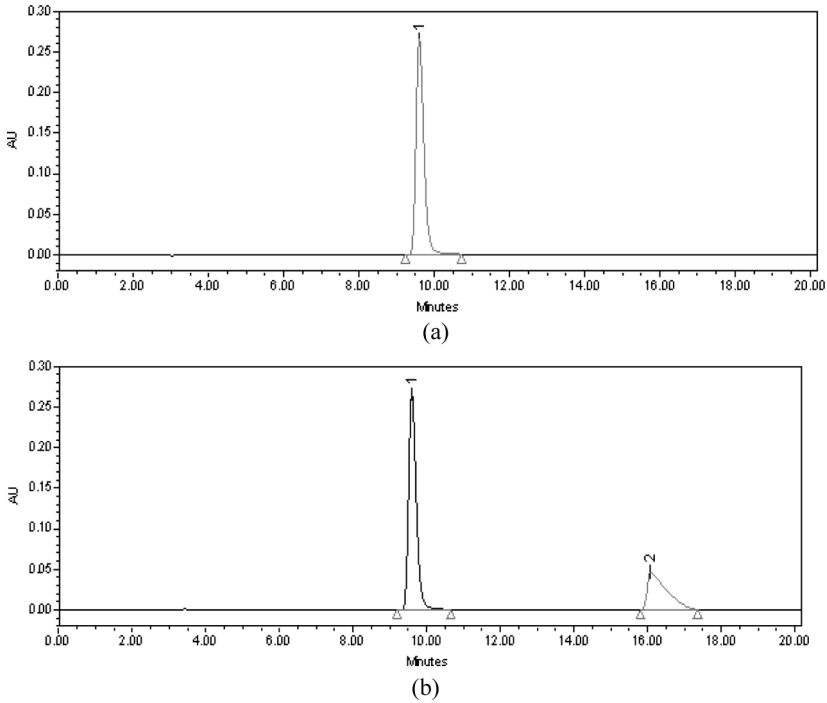

Figure 3. HPLC chromatogram of zoledronic acid at each concentration of 400 μg mL^{-1}, respectively for assay. (a) System suitability chromatogram for standard and (b) Sample chromatogram; Peaks: zoledronic acid, 1; placebo, 2.

Oxidation Studies

Solutions for use in oxidation studies were prepared in 10% hydrogen peroxide and the resultant solution heated on a water bath at 80°C for 6 hours was injected prior to analysis.

RESULTS AND DISCUSSION

Validation of Developed Stability Indicating Method

The proposed test method was validated to include requirements of (ICH) guidelines.

Parameters like system suitability, specificity, linearity, precision, accuracy, range, and ruggedness were examined.[13,14]

Table 2. System suitability parameters for related impurities and assay

Parameter	Imidazol	Imidazol-1-yl-acetic acid	Zoledronic acid	
			Impurities	Assay
Theoretical Plates[a]	6800	10200	6200	6150
Resolution	–	8.4	7.5	–
USP Tailing factor	1.33	1.20	1.25	1.60
[b]RSD (%)	1.26	0.88	1.75	0.26

[a]Per column length.
[b]For five replicate injections.

System Precision and System Suitability

System precision and system suitability for impurities was evaluated by injecting five times a mixed diluted solution comprised of imidazol, imidazol-1-yl-acetic acid, and zoledronic acid at $1.2\,\mu g\,mL^{-1}$ concentration. For assay a standard solution of zoledronic acid at about $400\,\mu g\,mL^{-1}$ was injected five times into the HPLC system. Method performance data including number of theoretical plates, USP tailing factor, resolution, and %RSD are listed in Table 2.

Specificity/Selectivity

Placebo Interferences

Placebo samples were prepared in triplicate by taking the placebo (excipients used in the drug product) equivalent to about the same weight in a portion of the sample preparation. The placebo solution did not show any peak at the retention time of imidazol, imidazol-1-yl-acetic acid, and zoledronic acid. This indicates that the excipients used in the formulation matrix do not interfere in the estimation of impurities and zoledronic acid. This is an important advantage of the present method over the reported methods.

Degradation Behavior of Zoledronic Acid

HPLC studies of samples obtained on forced decomposition studies of zoledronic acid under different conditions, and by using the above experimental conditions, suggested the following degradation behavior. The drug was found to be highly stable under 1.0 M HCl, 1.0 M NaOH, and neutral (water) conditions at 80°C for 6 hours. No major degradants

were observed in any of these conditions. The drug was found to be slightly labile to hydrogen peroxide at 80°C for 6 hours. It decomposed to an extent of 4%. The major degradants observed were well baseline resolved. Purity angle (PA) was less than purity threshold (TH) for all stressed and unstressed samples and the % assay for all the stressed samples was found to be >99%. Mass balance was calculated by summing up the % assay and % degradants and was found to be close to 100% at all the stressed conditions. Thus, this method is considered to be "Stability Indicating" (Figure 4).

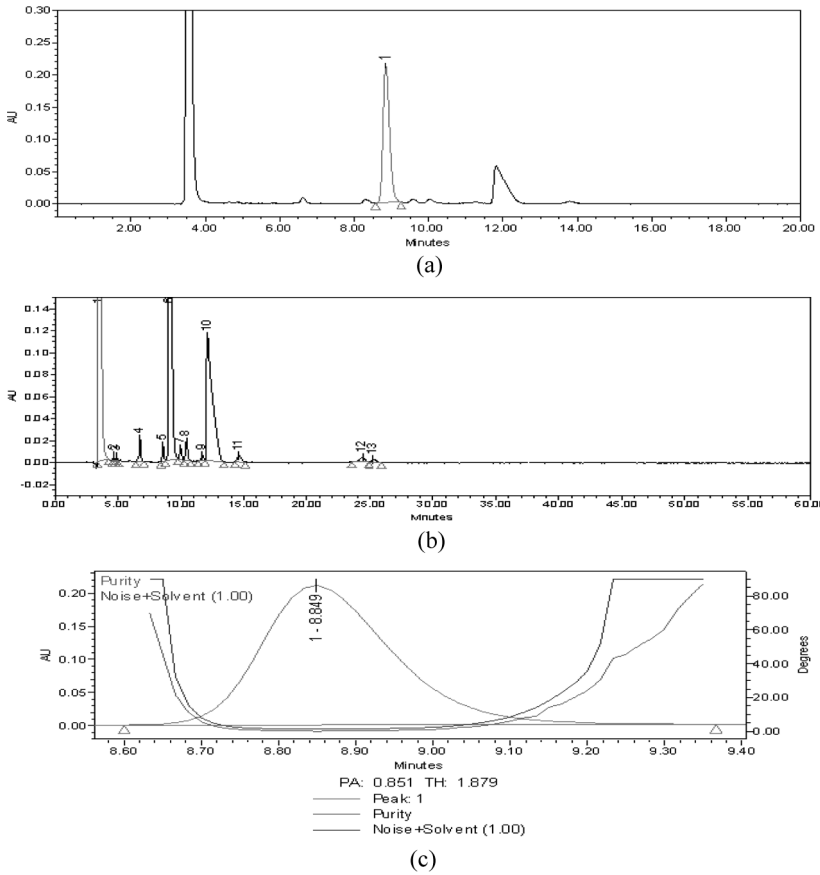

Figure 4. HPLC chromatogram of zoledronic acid under peroxide stress condition. (a) represents sample chromatogram for assay; Peaks: zoledronic acid, 1, (b) represents sample chromatogram for impurities showing the separation of zoledronic acid from other degradants; Peaks: hydrogen peroxide blank, 1; degradants, 2, 3, 4, 5, 7, 8, 9, 11, 12, 13; zoledronic acid, 6; placebo, 10, and (c) spurity plot of zoledronic acid.

Table 3. Repeatability and intermediate precision data evaluated through intra-day and inter-day studies

Compound	Level of addition	Actual ($\mu g\ mL^{-1}$) Concentration	[a]Measured concentration ($\mu g\ mL^{-1}$); ±S.D; RSD (%)	
			Intra-day	Inter-day
Zoledronic acid	100%	400	397.6 ± 0.98; 0.25	398.3 ± 0.89; 0.22
Imidazol	0.20%	0.800	0.800 ± 0.01; 1.25	0.787 ± 0.02; 2.54
Imidazol-1-yl-acetic acid	0.20%	0.800	0.793 ± 0.01; 1.26	0.780 ± 0.01; 1.28

[a](n = 6).

Method Precision (Repeatability) and Intermediate Precision (Reproducibility)

Method precision or intra-day precision for impurities was performed by spiking imidazol and imidazol-1-yl-acetic acid at $0.8\,\mu g\,mL^{-1}$ (0.2% as specification limit) in the presence of a placebo six times. For assay, Six replicate (n = 6) solutions were prepared and each solution was injected in duplicate under the same conditions, and mean value of peak area response for each solution were taken. Intermediate precision (inter-day precision) was performed by analyzing the study using a different instrument, analyst, column, and six different samples at the stated concentration. The results of repeatability and intermediate precision experiments are shown in Table 3. The results of the precision and intermediate precision study indicate that the method is reproducible and reliable.

Accuracy (Recovery Test)

Accuracy of the method for impurities was studied by recovery experiments performed by spiking imidazol and imidazol-1-yl-acetic acid from $0.3\,\mu g\,mL^{-1}$ to $2.0\,\mu g\,mL^{-1}$ in the sample. Three samples were prepared for each recovery level. Calculated average recovery and RSD for each level are tabulated in Table 4. The recovery for the assay was performed at three levels 50%, 100%, and 150% of the label claim (4 mg/vial). Three samples were prepared for each recovery level. The average % recovery values for 50%, 100%, and 150% was found to be 97.5, 99.5, and 99.9, respectively, and the average % recovery for nine determinations was 99.0 with %RSD 1.33.

Table 4. Accuracy (Recovery test)

Compound	Level of addition	Actual Concentration ($\mu g\ mL^{-1}$)	[a]Measured concentration ($\mu g\ mL^{-1}$); ±S.D; RSD (%)	Recovery (%)
Imidazol	0.1%	0.323	0.325 ± 0.0006; 0.18	100.5
	0.2%	0.808	0.808 ± 0.0006; 0.69	100.0
	0.3%	1.293	1.277 ± 0.0040; 0.32	98.7
	0.4%	1.616	1.559 ± 0.0040; 0.26	96.5
	0.5%	2.101	2.070 ± 0.0529; 2.56	98.5
Imidazol-1-yl-acetic acid	0.1%	0.297	0.284 ± 0.0038; 1.33	95.5
	0.2%	0.744	0.735 ± 0.0047; 0.64	98.8
	0.3%	1.190	1.167 ± 0.0082; 0.70	98.1
	0.4%	1.487	1.467 ± 0.0031; 0.21	98.6
	0.5%	2.082	2.072 ± 0.0056; 0.27	99.5
Zoledronic acid	50%	200	195 ± 2.00; 1.03	97.5
	100%	400	398 ± 2.00; 0.50	99.5
	150%	600	600 ± 4.73; 0.79	99.9

[a](n = 3).

Limit of Quantitation (LOQ)

LOQ for imidazol, imidazol-1-yl-acetic acid, and zoledronic acid were established by identifying the concentration, which gives signal-to-noise ratio (S/N) of about 10:1 and was found to be $0.11\,\mu g\,mL^{-1}$, $0.17\,\mu g\,mL^{-1}$, and $0.32\,\mu g\,mL^{-1}$, respectively. At LOQ level, precision was performed by spiking the above component in the presence of the placebo and the % RSD was found to be less than 5.0. The results are shown in Table 1.

Response Linearity and Range

The mixed seven standard solutions containing imidazol and imidazol-1-yl-acetic acid in the concentration range of LOQ to $4\,\mu g\,mL^{-1}$ for impurities and 40–$600\,\mu g\,mL^{-1}$ of zoledronic acid for assay, respectively, were prepared and injected in duplicate into the chromatographic system. In the simultaneous determination, the calibration curve showed good linearity over the concentration range. The correlation coefficient, regression equation between the peak area and the respective concentration values are depicted in Table 1.

Analyte Solution Stability (Ruggedness)

The stability of the standard and sample solutions was tested at intervals of 24 hours, 48 hours, and 72 hours on the bench top against the freshly

prepared standard solutions and the solutions were found to be stable up to 72 hours.

Application

To test the validity of the present method, the authors have used this method for the analysis of real commercial samples and the samples that were subjected to the accelerated stability conditions of 60°C for 3 months. The test solutions subjected to HPLC analysis showed no major degradants with total impurities <0.1% indicating the stability of the drug product.

Optimization of the Chromatographic Conditions

Design of the analytical method involves three critical steps such as chromatograph conditions (mobile phase and column), sample preparation (diluent effect and concentration), and detection mode (type of detector and injection volume). The chromatograph method was optimized by changing various variable parameters, one at a time, of the mobile phase like buffer concentration, ion pair concentration, and buffer pH.[15,16]

Effect of Buffer Concentration

The effect of buffer concentration in the mobile phase was studied on zoledronic acid peak symmetry by varying the amount of the buffer concentration from 20 mM to 40 mM in the mobile phase. From these studies (based on the USP tailing factor <2.0), 30 mM was found to be ideal buffer concentration in the mobile phase.

Effect of Ion Pair Reagent Concentration

The effect of ion pair concentration in the mobile phase was studied by varying the amount of tetra butyl ammonium hydrogen sulphate ion pair reagent concentration from 2 mM to 15 mM. From these studies, it was observed that at low concentration of ion pair reagent (<5 mM), retention time due to zoledronic acid peak decreased and the resolution between zoledronic acid and imidazol-1-yl-acetic acid was found to be less than 2.0. However, the retention times for the impurities remain unchanged during the above studies. Good resolution, peak symmetry, and retention times were observed at a concentration of 5 mM and above. From these studies, 5 mM concentration was chosen in the mobile phase. Although, the increase in the concentration of ion pair reagent resulted in a better peak shape, it was observed that as the concentration of ion pair

reagent was increased, a decrease in the column life (less than of about 40 injections) was observed.

Mobile Phase Selection

Experiments were carried out using 100%, 90%, and 80% aqueous buffered mobile phase to retain zoledronic and its impurities on the stationary phase. It was observed that at 90% aqueous buffer composition in the mobile phase, the peak retention times of imidazol (about 4.9 minutes), imidazol-1-yl-acetic acid (about 7.1 minutes), zoledronic acid (about 10.2 minutes), and placebo peak (about 14 minutes) were observed on a C_{18} column, showing good resolutions between impurities, zoledronic acid, and the excipients used in the formulations. Also, 10% of organic phase in the mobile phase helps to prevent the column phase collapse, increasing the life of the column.

Effect of Buffer pH

Since zoledronic acid is polar and acidic, a correct suitable pH choice is to be made in order to have a good effect of ion pair and retention. An acidic compound should be ionized completely so that the free acidic groups in the molecule in hydrophilic state binds completely with the positively charged ion pair (reverse engineering effect), leading to more retention on non-polar columns; therefore, pH of the buffer was varied from 6.8 to 7.8. From these studies, it was observed that the tailing factor for zoledronic acid decreased with the increase in pH, but at pH 7.6 and above, the resolution between zoledronic acid and placebo peak was decreased (<2.0). However, there was no significant change in the retention times of impurities, which indicates impurities are a neutral form, while the retention time for the peak due to zoledronic acid increased as the pH was increased, indicating the impact of ionization of an acidic compound. Based on these studies and peak symmetry (USP Tailing factor <2.0), the optimum pH value for the buffer was found to be in the range of 7.0 to 7.4 and the method was found to be robust at these pH conditions.

Wavelength Selection

Zoledronic acid and its related impurities shows UV absorption maxima at about 210 nm. The major degradant obtained during the stress studies was found to be imidazol-1-yl-aectic acid under peroxide stress conditions. The wavelength 215 nm was selected to avoid mobile phase and placebo interferences at the low wavelength.

Column Selection

Zoledronic acid is highly polar and acidic in nature and it elutes in less than 3 minutes on C_8 or C_{18} column. Because of the polar nature of the molecule, the proposed method mobile phase conditions were used for the column study. Under these mobile phase conditions, retention time for zoledronic acid on the C_8 column was found to be less than 6 minutes with poor resolution (<2.0), while a reasonable retention time of about 11 minutes and good resolution (>7.0) was observed on the C_{18} column. Early retention times observed on the C_8 column did not yield a good baseline separation of impurities and degradants observed under peroxide stress conditions. However, good baseline separations were achieved on the C_{18} column, which is the added advantage of this method over the reported methods.

CONCLUSIONS

The IP-HPLC method developed meets the system suitability criteria, peak integrity, and resolution for the parent drug (zoledronic acid) and its impurities and degradants. Low quantification limits achieved describe the method as very sensitive, which is the added advantage over the reported methods. High recoveries and acceptable RSD values confirm established the IP-HPLC method is suitable in quality control for zoledronic acid injection. The IP-HPLC method can be applied as a stability indicating method for zoledronic acid injection. Possible temperature and humidity fluctuations, outside of labeled storage conditions during transportation of zoledronic acid injection, can be evaluated using the reported IP-HPLC method.

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