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ORIGINAL ARTICLE

A simple RP-HPLC method for related substances of zoledronic acid in pharmaceutical products

L. Maheswara Reddy [a], K. Janardhan Reddy [b,*], P. Raveendra Reddy [a]

[a] *Department of Chemistry, Sri Krishnadevaraya University, Anantapur 515 055, Andhra Pradesh, India*
[b] *Department of Nanomaterial Chemistry, College of Science & Technology, Dongguk University, 707 Seokjang-Dong, Gyeongju, Gyeongbuk 780 714, Republic of Korea*

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UV-detector;
Zoledronic acid recovery studies

Abstract A novel, selective and sensitive reverse phase-high performance liquid chromatography (RP-HPLC) method has been developed for the validated estimation of imidazol-1-yl-acetic acid in zoledronic acid formulations. The separation was achieved on a 5 μ C18 column (250 × 4.6 mm) using a mobile phase that consists of the buffer (4.5 g of di-potassium hydrogen phosphate anhydrous and 2.0 g of tetra butyl ammonium hydrogen sulphate (TBAHS) in 1000 mL of water) and methanol in the ratio of 900:100 v/v. The flow rate was maintained at 1.0 mL min^{-1}. The detection of the constituents was done at 215 nm using a UV detector. The retention times of imidazol-1-yl-acetic acid and zoledronic acid were 7.2 and 10.2 min respectively. Recovery studies were satisfactory and the correlation coefficient, 0.999 indicates linearity of the method within the limits. The developed method can be applicable for regular qualitative analysis.

1. Introduction

Zoledronic acid is a nitrogen containing bisphosphonic acid, widely used to prevent or treat osteoporosis in post-menopausal women, in men or women who have taken glucocorticoids (a type of corticosteroid medication that may cause osteoporosis), paget's disease of the bone (a condition in which the bones are soft and weak, and may be deformed, painful or easily breakable), high levels of calcium in the blood may occur with certain types of cancer, due to its high potential and inhibits osteoclastic bone resorption (Adami et al., 2002; Amanat et al., 2007; Andrew et al., 2008; Aparicio et al., 1998; Berenson, 2005; Black et al., 2007; Body et al., 1999; Boutsen et al., 2001; Conte and Guarneri, 2004).

The literature survey reveals that HPLC and RP-HPLC for determination of zoledronic acid are reported (Jiang et al., 2004; Mallikarjuna Rao et al., 2005; Praveen Kumar and Sreeramulu, 2011; Srinivasan Raghu Nandan et al., 2009; Jiang et al., 2005; Zhang et al., 2004; Matuszewski et al., 2011). But, a few are (Praveen Kumar and Sreeramulu, 2011; Srinivasan Raghu Nandan et al., 2009; Zhang et al., 2004) about stability indicating reverse phase high-performance liquid chromatography (RP-HPLC) methods for the determination

* Corresponding author. Tel.: +82 54 770 2215; fax: +82 54 770 2386.
E-mail address: reddyjchem@gmail.com (K. Janardhan Reddy).
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of zoledronic acid in pharmaceutical injections/tablets. Even for though those who are suffering from interference of impurities/intermediates when forced to stress conditions the method becomes less sensitive with low selectivity. Therefore, the development of a new economical, selective and sensitive RP-HPLC method for zoledronic acid and its related substances is intended.

The main objective of this study was to develop a novel, simple, economical, selective, sensitive and stable method indicating the use of reverse phase-high performance liquid chromatography (RP-HPLC) method for the assay of zoledronic acid and its related substances, imidazol-1-yl-acetic acid (Fig. 1) present in pharmaceutical products using a UV detector. It was included in the performance of stress/influenced factors, such as acid or base hydrolysis, hydrogen peroxide oxidation, light and heat on zoledronic acid and its related substances, inimidazol-1-yl-acetic acid determination. This paper also deals with the validity of the proposed method for the accurate estimation of zoledronic acid and related substances in pharmaceutical samples. The mobile phase was used as diluent for the preparation of working solutions, which minimizes errors that occur in quantitative separation techniques. This proposed method was successfully applied for regular analysis.

2. Materials and methods

2.1. Reagents

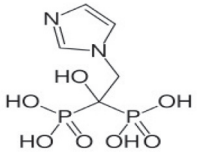
Dipotassium hydrogen phosphate anhydrous (Merck, GR Grade), tetra butyl ammonium hydrogen sulphate (TBAHS) (Merck, German, Catalogue. No. 8.18858.0100/synthesis grade), methanol (HPLC grade), water (HPLC grade), phosphoric acid 85% pure (Merck grade) was used in the present study. All other chemicals used were of analytical reagent grade, otherwise stated.

2.2. Chromatographic system conditions

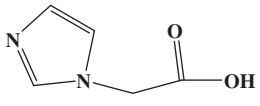
Kromosil 5 μ C18 column (250 × 4.6 mm) at 323 K oven temperature for 45 min (for blank, sample preparation), and 15 min (for standard preparation) run time with 1.0 mL min^{-1} flow rate was used for the separation of impurities in zoledronic acid or related substances and detected at 215 nm using a UV detector. 15 μL of sample was used for the injection into the column. Water and mobile phases were used as diluents.

2.3. Preparation of mobile phase

Accurately weighed 4.5 g of dipotassium hydrogen phosphate anhydrous and 2.0 g of tetra butyl ammonium hydrogen sulphate in a 1000 mL glass beaker, added 1000 mL of water, and sonicated for 10 min on occasional stirring with a glass rod. Well mixed buffer and methanol in the ratio of 900:100 v/v was used as the mobile phase.



Zoledronic acid



Imidazol-1-yl-acetic acid

Figure 1 Chemical structure of zoledronic acid and impurity (Imidazol-1-yl-acetic acid).

2.4. Impurity (20 μg mL^{-1}) and standard (32 μg mL^{-1}) stock solution preparation

Accurately weighed and transferred 2.0 mg of imidazol-1-yl-acetic acid impurity into a 100 mL volumetric flask, dissolved 70 mL of the mobile phase and made up to the mark with mobile phase and used as impurity stock.

Weighed accurately about 21.4 mg of zoledronic acid monohydrate (equivalent to 20.0 mg of zoledronic acid) into a 25 mL volumetric flask, added about 20 mL of mobile phase, sonicated to dissolve for about 15 min with occasional shaking and diluted to volume with mobile phase and mixed well, which is used as a stock standard. Pipette out 1.0 mL of the above solution into a 25 mL volumetric flask and dilute to volume with mobile phase.

2.5. Standard and test solution preparation

Pipette out 2.0 mL each of the above standard stock solution and impurity stock solution into a 25 mL volumetric flask and made up with diluents for making a standard solution.

Opened five vials (each vial contains zoledronic acid: 4 mg 5 mL^{-1}). Pooled the content of each individual vial into a clean and dried 50 mL volumetric flask or into a dried 25 mL stoppered test tube and mixed well with diluents. This solution was used as test solution for further studies.

2.6. System suitability

The relative standard deviation for zoledronic acid or imidazol-1-yl-acetic acid peak area and peak area of six replicate injections should not be more than 10.0%; The tailing factor for zoledronic acid and imidazol-1-yl-acetic acid peaks should not be more than 2.0; The column efficiency (theoretical plates) for zoledronic acid and imidazol-1-yl-acetic acid peaks that should be not less than 2000 is necessary for good system suitability. For good system suitability to determine imidazole-1-yl-acetic acid less than 10% RSD, less than tailing factor and more than 2000 theoretical plates were maintained.

The presence of imidazol-1-yl-acetic acid content in the pharmaceutical samples was calculated using the following formula.

% Of imidazol-1-yl-acetic acid:

$$\frac{A \times W_i \times 2 \times P_i}{S_i \times 100 \times 25 \times 0.8}$$

% Of any unknown impurity:

$$\frac{B \times \mathrm{Ws} \times 1 \times 2 \times P_s}{\mathrm{Ss} \times 25 \times 25 \times 25 \times 0.8}$$

where, A = peak area of imidazol-1-yl-acetic acid for test preparation; B = peak area of any impurity other than blank, placebo, imidazol-1-yl-acetic acid and zoledronic acid for test preparation, W_i = weight of imidazol-1-yl-acetic acid impurity taken in mg for standard preparation; Ws = Weight of zoledronic acid monohydrate working standard taken in mg for

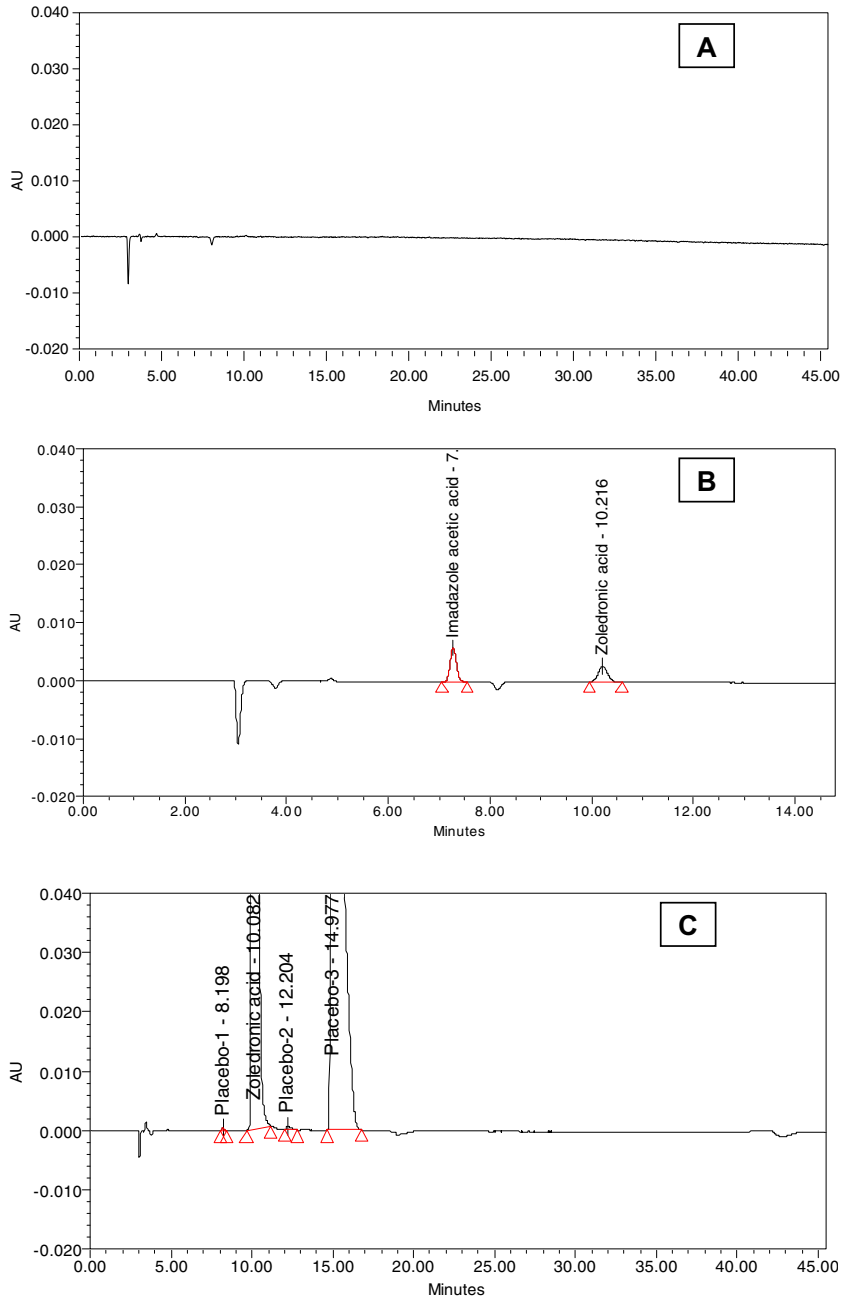


Figure 2 (A) Blank/diluents, (B) standard solution and (C) test solution chromatogram.

Table 1 Study of system precision.

Injection Number	Zoledronic acid peak area	Imidazol-1-yl-acetic acid peak area	Acceptance criteria
01	29270	43563	The relative standard deviation of peak areas of zoledronic acid and imidazol-1-yl-acetic acid peak area should not be more than 10.0%.
02	28683	43546	
03	28505	43575	
04	28101	43621	
05	27747	43396	
06	27329	43474	
Mean	28272	43529	
%RSD	2.5	0.2	

Table 2 Study of system suitability.

System suitability for standard preparation		Observed value	Acceptance criteria
The tailing factor for peaks	Zoledronic acid	1.09	Should not be more than 2.0
	Imidazol-1-yl-acetic acid	1.10	
The column efficiency for standard peaks	Zoledronic acid	13788	Should not be less than 2000
	Imidazol-1-yl-acetic acid	16278	

Table 3 Forced degradation studies on zoledronic acid.

Stress condition	Drug product			
	% Degradation	Zoledronic acid		
		Purity angle	Purity threshold	Purity flag
Acid degradation (1.0 mol L^{-1} HCl at 353Kon water bath for 6 h)	0.0	0.447	1.393	NO
Base degradation (1.0 mol L^{-1} NaOH at 353 K on water bath for 6 h)	0.0	0.706	1.476	NO
Peroxide degradation (10% H_2O_2 at 353 K on water bath for 6 h)	1.91	0.455	1.309	NO
UV degradation ($\geqslant$200 wh/m^2 at 298 K with UV radiation at 320–420 nm)	0.0	0.352	1.299	NO
Thermal degradation (363 K for 6 h)	0.0	0.506	1.401	NO
Sunlight degradation (24 h)	0.0	0.450	1.326	NO
Un stressed sample	0.0	0.445	1.347	NO

standard preparation; P_i = Purity of imidazol-1-yl-acetic acid impurity in %; P_s = Potency of zoledronic acid monohydrate working standard as zoledronic acid in %; S_i = Peak area of imidazol-1-yl-acetic acid for diluted standard preparation; S_s = Peak area of zoledronic acid for standard preparation; 0.8 = Label claim of zoledronic acid in mg mL^{-1}.

3. Results and discussion

3.1. System precision and suitability

A standard solution was prepared by mixing the working standard (zoledronic acid monohydrate) and imidazol-1-yl-acetic acid impurity as per the test method and was injected six times into the HPLC system. The system suitability parameters were evaluated from the standard chromatogram for both zoledronic acid and imidazol-1-yl-acetic acid and the results are illustrated in Fig. 2. The system suitability was found to be within the limits. Fig. 2 represents the diluents, standard solution and test solution chromatograms. System suitability results and precision results are also tabulated in Tables 1 and 2. The obtained results were within the limits, indicating that the system is more precise with good suitability.

3.2. Specificity

3.2.1. Placebo interference

A study to establish the interference of placebo was performed. Placebo solution was injected in triplicate as per the test method. Chromatogram of placebo did not show any peak at the retention time of zoledronic acid and imidazol-1-yl-acetic acid. This indicates that the excipients used in the injection formulation (zoledronic acid injection 4.0 mg 5 mL^{-1}) do not interfere in the estimation of related substances of zoledronic acid.

3.2.2. Interference from degradation products

A study was conducted to demonstrate the effective separation of degradants/impurities from zoledronic acid. Separate portions of sample bulk solution and placebo solutions were exposed to the following stress conditions to induce degradation. Stressed and unstressed samples were injected into the HPLC system with photo diode array detector by following test method conditions. All degrading peaks were resolved from zoledronic acid peak in the chromatograms of all samples and placebo did not show any interference at the retention time of zoledronic acid and imidazol-1-yl-acetic acid under the above conditions. Degradation study results were presented in Table 3 and chromatograms were shown in Fig. 3. From the results, the determination of zoledronic acid was not affected with any stress/influenced factor, but moderately affected with the oxidation conditions, especially, 10% hydrogen peroxide at 353 K oxidation of zoledronic acid shifted its peak to a lower retention time from its regular one (Fig. 3(C)). The remaining forced conditions were not affected bythe retention time and peak area of zoledronic acid and its related substances.

3.3. Precision and accuracy of the test method

The precision of the method was evaluated by analysing six samples which were prepared as per the test method by spiking imidazol-1-yl-acetic acid at 0.2% of target test concentration (800 μg mL^{-1}) in the presence of sample bulk solution. The percentage of relative standard deviation for imidazol-1-yl-acetic acid was calculated and the results were found to be within the limits (Table 4). Hence, the results indicated that the method was more precise.

An accuracy study of imidazol-1-yl-acetic acid from spiked samples of zoledronic acid was conducted. Samples were prepared in triplicate at each level by spiking imidazol-1-yl-acetic acid in the presence of sample bulk solution at 50%, 75%, 100%, 125%, and 150% to the target concentration (0.2%). The percentage recovery and average percentage recovery for impurity was found to be within the limits and the results are shown in Table 5.

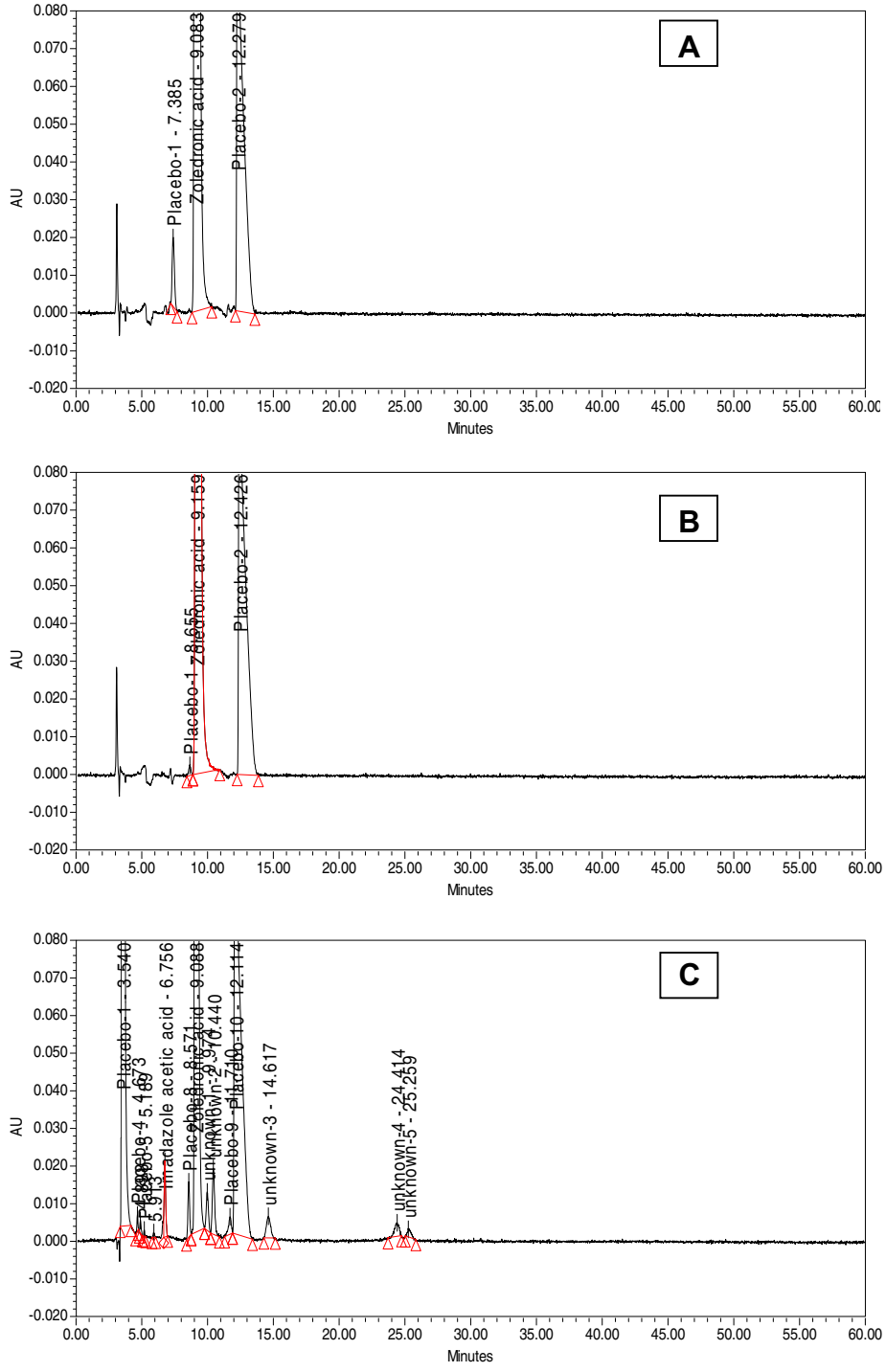


Figure 3 Forced degradation/stability studies of zoledronic acid with (A) acid stressed, (B) base stress, (C) peroxide stress, (D) UV light stress, (E) thermal stress, (F) sun light stress, (G) un stressed chromatogram.

3.4. Linearity of the test method

A linearity graph was plotted for average '$\mu g\ mL^{-1}$' of imidazol-1-yl-acetic acid added against average '$\mu g\ mL^{-1}$' of imidazol-1-yl-acetic acid found in the accuracy section. The correlation coefficient (0.999) was found to be within the limits indicating that the test method was linear for test sample determinations, which is shown in Fig. 4.

3.5. Limit of detection (LOD) and limit of quantification (LOQ)

A study to establish the limit of detection and limit of quantification of imidazol-1-yl-acetic acid was conducted. Limit of detection (LOD) and limit of quantification (LOQ) were established based on the signal to noise ratio. A series of solutions having different concentrations (range) of imidazol-1-yl-acetic

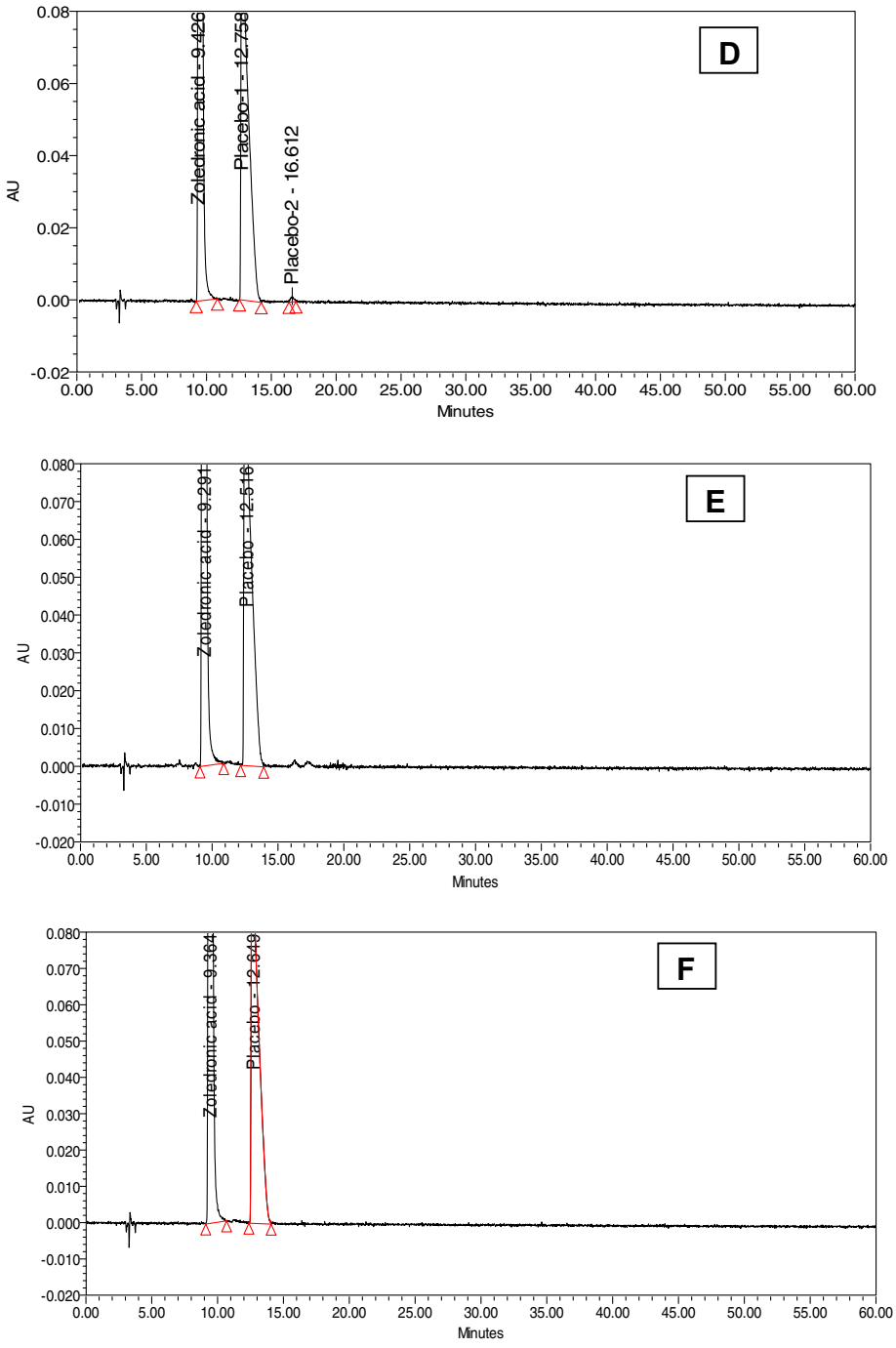


Fig. 3 (*continued*)

acid were prepared by spiking the impurity in the presence of sample bulk solution and were injected. Limit of detection was established by identifying the concentration, which gives signal to noise ratio of about 3.0. Limit of quantification for the impurity was established by identifying the concentration, which gives signal to noise ratio of about 10. For testing LOD and LOQ six samples were prepared by spiking imidazol-1-yl-acetic acid (0.04 μg mL^{-1} and 0.16 μg mL^{-1} for testing LOD and LOQ respectively) to the solution which was prepared in precision and accuracy of impurity determination experiments at about the limit of quantification and detection in the presence of sample bulk solution as per the test method and were injected into HPLC. The obtained results found 0.005% and 0.02% at LOD and LOQ with 3.5 and 14.0 signal to noise ratio respectively.

3.6. Linearity of detector response

A linearity study of detector response (Area) versus concentration (μg mL^{-1}) of imidazol-1-yl-acetic acid was conducted. A series of solutions of imidazol-1-yl-acetic acid in the concentration range of about limit of detection quantity (LOQ) level to 200% of target concentration (0.2%) were prepared and injected into the HPLC system. The detector response was found

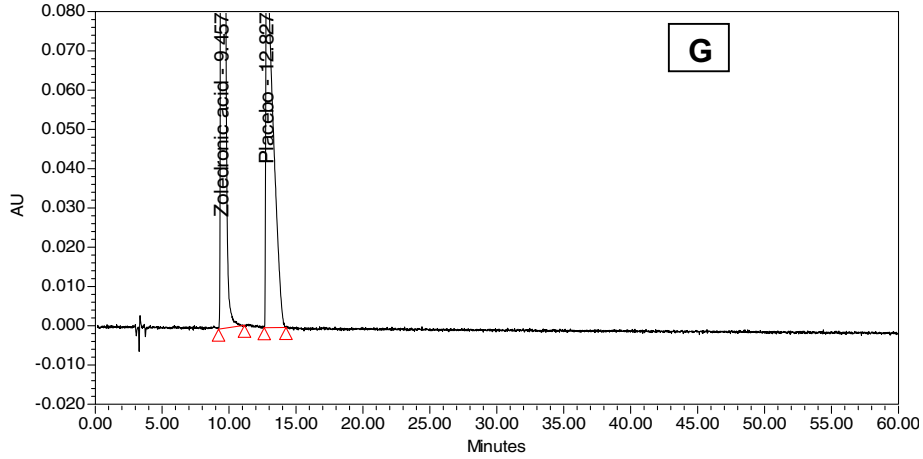


Fig. 3 (*continued*)

Table 4 Precision study of the method.

Sample	RRT	Imidazol-1-yl-acetic acid	
		Peak area	% Imp
01	0.75	43476	0.195
02	0.74	43701	0.196
03	0.74	43665	0.195
04	0.74	43621	0.195
05	0.75	43787	0.196
06	0.75	44170	0.198
Average	0.75	–	0.196
%RSD	–	–	0.59

to be linear from LOQ to 200% of the target concentration and the correlation coefficient was found to be within the limits.

3.7. Range, precision and accuracy

A study of range for imidazol-1-yl-acetic acid was conducted for precision, accuracy and linearity. The range of the test method was evaluated by analysing the following parameters.

Samples were prepared six times by spiking imidazol-1-yl-acetic acid at the limit of quantification (LOQ) and 150% of target concentration (0.2%) and were injected into the HPLC system. The calculated relative standard deviation for the impurity at the limit of detection quantity (LOQ) level with 150% target spiked level. The % of RSD of individual impurities at LOQ level with 150% spiked level was found to be within the limits.

Samples were prepared in triplicate at lower spiked level (LOQ level) with highest spiked level (150%) to the target concentration of imidazol-1-yl-acetic acid. The percentage of recoveries and average percentage of recoveries were found to be within the limits.

3.8. Ruggedness of the test method

3.8.1. System to system/analyst to analyst/column to column variability

System to system/analyst-to-analyst/column to column variability study was conducted on different HPLC systems, different columns, on different days and by different analysts under similar conditions at different times. Six samples were prepared as per the test method by spiking imidazol-1-yl-acetic acid at 0.2% of the target test concentration (800 μg mL^{-1})

Table 5 Recovery results of imidazol-1-yl-acetic acid from zoledronic acid.

Sample No.	Spike level (%)	Added, 'μg mL^{-1},	Recovered, 'μg mL^{-1},	% Recovery	Mean % Recovery
1	50	0.814	0.838	102.9	103.2
2	50		0.842	103.4	
3	50		0.841	103.3	
1	75	1.221	1.259	103.1	102.9
2	75		1.252	102.5	
3.	75		1.258	103.0	
1	100	1.629	1.627	99.9	100.2
2	100		1.635	100.4	
3	100		1.636	100.4	
1	125	2.036	2.128	104.5	104.4
2	125		2.123	104.3	
3	125		2.126	104.4	
1	150	2.443	2.497	102.2	102.1
2	150		2.482	101.6	
3	150		2.504	102.5	

in the presence of sample bulk solution. The percentage of relative standard deviation for imidazol-1-yl-acetic acid was calculated and the results were found to be within the limits. Comparison of both the results obtained on two different HPLC systems, different columns and different analysts shows that the related substances test method is rugged for system to system/analyst to analyst/column to column variability.

3.8.2. Bench top stability of standard and test preparation

A study to establish the analytical solution stability of the standard and test preparations on bench top was conducted over a period of 48 h. Standard preparation and test preparation with impurities spiked at target concentration were injected at initial, 24 and 48 h. The difference in the % of known individual impurities and the % of total impurities from initial to 48 h was within the limits. From the above study, it was established that the standard and sample solutions were stable for 48 h on bench top.

3.8.3. Bench top stability of mobile phase

A study to establish the bench top stability of the mobile phase for a period of about 2 days was conducted. Standard preparation and test preparation with impurities spiked at target concentration were injected at initial, 24 and 48 h. The difference in the percentage of known individual impurities and the percentage of total impurities from initial to 48 h was within the limits. From the above study, it was established that the mobile phase was stable for 48 h on bench top.

3.9. Robustness

3.9.1. Effect of variation of flow rate

A study was conducted to determine the effect of variation in the flow rate. Standard solution prepared as per the test method was injected into the HPLC system using flow rates of 0.6 and 1.0 mL min^{-1} instead of 0.8 mL min^{-1}. The system suitability parameters were evaluated and found to be within the limits for 0.6 and 1.0 mL min^{-1} flow. Test solution was prepared as per the test method by spiking imidazol-1-yl-acetic acid at 0.2% of target concentration (800 μg mL^{-1}) in the presence of sample bulk solution and was injected into the HPLC system using flow rates of 0.6 and 0.8 mL min^{-1} flow. Imidazol-1-yl-acetic acid was well resolved from all other peaks and the relative retention times were comparable with those obtained for the mobile phase having flow rates of 0.6 and 1.0 mL min^{-1}. From the above study it was established that the allowable variation in flow rates is 0.6 and 1.0 mL min^{-1}.

3.9.2. Effect of column oven temperature variation

A study was conducted to determine the effect of variation in column temperature. Standard solution prepared as per the test method was injected into the HPLC system by thermo setting the column oven temperature at 318 and 328 K instead of 323 K. The system suitability parameters were evaluated and found to be within the limits for 318 and 328 K. Test solution was prepared as per the test method by spiking imidazol-1-yl-acetic acid at 0.2% of the target concentration (800 μg mL^{-1}) in the presence of sample bulk solution and was injected into the HPLC system, keeping the column oven temperatures at 318 and 328 K. Imidazol-1-yl-acetic acid was well resolved from all other peaks and the relative retention times were comparable with those obtained for the column temperatures 318 and 328 K. From the above study it was established that the allowable variation in column oven temperature is 318–328 K.

3.9.3. Effect of variation in the mobile phase composition

A study was conducted to determine the effect of variation of mobile phase composition. Prepared standard solution was injected into the HPLC system using two different mobile phases. The system suitability parameters were evaluated and found to be within the limits for the mobile phase having 90% and 110% of the method organic phase. Test solution was prepared as per the test method by spiking imidazol-1-yl-acetic acid at 0.2% of target concentration (800 μg mL^{-1}) in the presence of the sample bulk solution and was injected into the HPLC system using a mobile phase having 90% and 110% of the method organic phase separately. Imidazol-1-yl-acetic acid was well resolved from all other peaks and the relative retention times were comparable with those obtained from the actual method. From the above study it was established that the allowable variation in organic phase composition in the mobile phase is 90% to 110% of the method organic phase of the mobile phase.

3.9.4. Effect of variation of pH

A study was conducted to determine the effect of variation in pH. Standard solution prepared as per the test method was injected into the HPLC system using the mobile phase at pH 7.0 and 7.4. The system suitability parameters were evaluated and found to be within the limits for 7.0 and 7.4. Test solution was prepared as per the test method by spiking imidazol-1-yl-acetic acid at 0.2% of target concentration (800 μg mL^{-1}) in the presence of sample bulk solution and was chromatographed using mobile phase at pH 7.0 and 7.4. Iimidazol-1-yl-acetic acid was well resolved from all other peaks and the relative retention times were comparable with those obtained for mobile phase having pH 7.0 and 7.4. From the above study it concluded that the allowed variation in the mobile phase pH is 7.0 and 7.4.

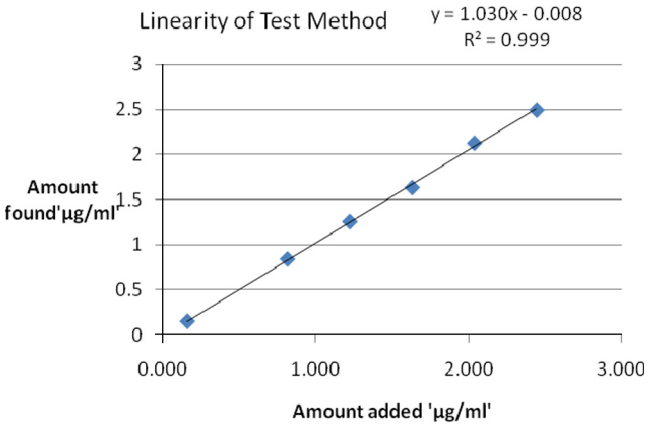


Figure 4 Linearity graph of imidazol-1-yl-acetic acid.

3.10. Advantages of the present RP-HPLC method

The present method is highly precise and accurate when compared with other existing RP-HPLC methods (Srinivasan Raghu Nandan et al., 2009). The present method can be performed more effectively even in the presence of degradation/stress factors, such as UV-light, sun light and high temperature conditions, except hydrogen peroxide for the separation and determination of zoledronic acid and its related substance, Iimidazol-1-yl-acetic acid (Jiang et al., 2004, 2005; Zhang et al., 2004), even though, it also showed good performance with high resolution up to 10% hydrogen peroxide without any impurity flag. The resolution of the zoledronic acid peak in the present method is 3.0 from its adjacent peaks, which was more than the reported methods (Jiang et al., 2004, 2005; Mallikarjuna Rao et al., 2005; Praveen Kumar and Sreeramulu, 2011; Zhang et al., 2004). Iimidazol-1-yl-acetic acid was well resolved from all other peaks and the relative retention times were comparable with those obtained for mobile phases having pH 7.0 and 7.4.The present method is more economical when compared with other existing methods where there is more chemical consumption (Matuszewski et al., 2011). The mobile phase itself is used as a diluent in the present method, which has minimized errors that occur in quantitative analysis.

4. Conclusion

The above RP-HPLC analytical method satisfies all validation parameters like system suitability, precision, specificity, accuracy, linearity of detector response, ruggedness (Analyst to analyst, system to system and column to column variability, bench top/ refrigerator stability) and robustness (mobile phase composition variation, variation in flow rate, temperature variation). At the same time the method satisfies the forced degradation study. It indicates that the method is more stable and suitable for the zoledronic acid and its related substances determination. Hence, the method can be used for the estimation of zoledronic acid injection 4 mg 5 mL^{-1} in regular analysis.

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