Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Pharmaceutical and Biomedical Analysis 39 (2005) 781–790
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

A validated stability indicating ion-pair RP-LC method for zoledronic acid

B. Mallikarjuna Rao [a,*], M.K. Srinivasu [a], Ch. Prathima Rani [a], S. Siva kumar [a], P. Rajender Kumar [b], K.B. Chandrasekhar [c], M. Veerender [d]

[a] *Analytical Research, Custom Pharmaceutical Services, Dr. Reddy's Laboratories, Hyderabad 500049, India*
[b] *Project Management, Custom Pharmaceutical Services, Dr. Reddy's Laboratories, Hyderabad 500049, India*
[c] *Department of Chemistry, Jawaharlal Nehru Technological University, College of Engineering, Anantapur 515002, India*
[d] *Process Research, Custom Pharmaceutical Services, Dr. Reddy's Laboratories, Hyderabad 500049, India*

Received 22 November 2004; received in revised form 17 April 2005; accepted 18 April 2005
Available online 25 May 2005

Abstract

The present paper describes the development of a stability indicating high performance liquid chromatographic (HPLC) assay method for zoledronic acid in the presence of its impurities and degradation products generated from forced decomposition studies. The drug substance was subjected to stress conditions of hydrolysis, oxidation, photolysis and thermal degradation. The degradation of zoledronic acid was observed under oxidative stress at higher temperature. The drug was found to be stable in other stress conditions attempted. Successful separation of the drug from the degradation products formed under stress conditions was achieved on a C18 column using a mixture of phosphate buffer that contains 7 mM tetra butyl ammonium hydrogen sulphate, an ion-pairing agent and methanol (95:5) as mobile phase. The developed HPLC method was validated with respect to response function, precision, accuracy, specificity and robustness. The developed HPLC method to determine the related substances and assay determination of zoledronic acid can be used to evaluate the quality of regular production samples. It can be also used to test the stability samples of zoledronic acid.
© 2005 Elsevier B.V. All rights reserved.

Keywords: RP-LC; Forced degradation; Validation; Solution and mobile phase stability; ICH

1. Introduction

Zoledronic acid, a bisphosphonic acid, is an inhibitor of osteoclastic bone resorption. Zoledronic acid is designated chemically as (1-hydroxy-2-imidazol-1-yl-phosphonoethyl) phosphonic acid monohydrate. Zoledronic acid is a white crystalline powder. Zometa (zoledronic acid) injection is the registered product of Novartis Pharmaceutical Corporation (innovator), which is commercially available in vials as a sterile liquid concentrate solution for intravenous infusion. Each 5-ml vial contains 4.264 mg of zoledronic acid monohydrate, corresponding to 4 mg zoledronic acid on an anhydrous basis. The marketing approval for zoledronic acid in Europe and US was given in the year 2002.

Stress testing is a part of developmental strategy under the ICH requirements and is carried out under more severe conditions than accelerated conditions. These studies serve to give information on drug's inherent stability and help in the validation of analytical methods to be used in stability studies [1–3]. A high performance liquid chromatographic (HPLC) method was reported in the literature for the analysis of zoledronic acid and its related substances by ion-pair RP-LC [4]. A highly sensitive RIA method for the determination of zoledronic acid in human serum, plasma and urine was also reported in the literature [5]. So far, to our present knowledge, no stability indicating HPLC method for zoledronic acid was published in any journals neither by innovator nor by any other manufacturer. Attempts were made to

* Corresponding author. Tel.: +91 40 23045440; fax: +91 40 23044044.
E-mail addresses: bmrao@drreddys.com, bmrao67@rediffmail.com (B.M. Rao).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.04.012

develop a stability indicating HPLC method for the related substance determination and quantitative estimation of zoledronic acid. This paper mainly deals with the forced degradation of zoledronic acid under stress conditions like water hydrolysis, acid hydrolysis, base hydrolysis, oxidation, heat and light. This paper also deals with the validation of the developed method for the accurate quantification of impurities and assay of zoledronic acid in bulk samples.

2. Experimental

2.1. Chemicals

Samples of zoledronic acid and its two impurities namely Imp-1 (imidazole) and Imp-2 (imidazol-1-yl acetic acid) (Fig. 1) were received from Process Research Department of Custom Pharmaceutical Services of Dr. Reddy's Laboratories Limited, Hyderabad, India. HPLC grade methanol and tetra-*n*-butyl ammonium hydrogen sulphate were purchased from Merck, Darmstadt, Germany. Analytical reagent grade di-potassium hydrogen orthophosphate and di-sodium hydrogen orthophosphate were purchased from Qualigens Fine Chemicals, Mumbai, India. High pure water was prepared by using Millipore Milli Q plus purification system.

2.2. Equipment

The HPLC system, employed in the method development, forced degradation studies and assay method validation was Agilent 1100 series LC system with a diode array detector. The output signal was monitored and processed using Chemstation software (Agilent) on Pentium computer (Digital Equipment Co.).

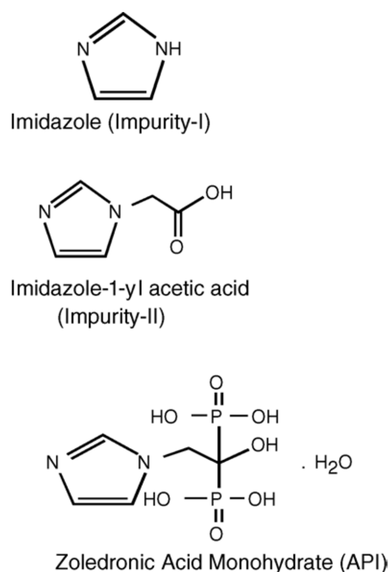


Fig. 1. Chemical structures of zoledronic acid, Imp-1 and Imp-2.

2.3. Chromatographic conditions

The chromatographic separation was achieved on a Waters X Terra RP18, 250 mm × 4.6 mm, 5 μm column using a mobile phase containing a mixture of aqueous 8 mM dipotassium hydrogen orthophosphate, 2 mM di-sodium hydrogen orthophosphate and 7 mM tetra-*n*-butyl ammonium hydrogen sulphate (pH of mixture is about 6.6) and methanol (95:5, v/v). The flow rate of the mobile phase was 0.7 ml/min. The column temperature was maintained at 25 °C and the wavelength was monitored at 215 nm. The injection volume was 10 μl. The test concentration used for the related substance analysis is 1.0 mg/ml and for assay 0.2 mg/ml. The standard and test dilutions were prepared in mobile phase. The total run time for each LC run for related substances determination is 45 min and assay estimation is 15 min.

2.4. Preparation of standard solutions

A stock solution of zoledronic acid (2.0 mg/ml) was prepared by dissolving appropriate amount of drug substance in mobile phase. Working solutions of 1000 and 200 μg/ml were prepared from stock solution for related substances determination and assay determination, respectively. A stock solution of impurity (mixture of Imp-1 and Imp-2) at 0.5 mg/ml was also prepared in mobile phase.

2.5. Specificity

Specificity is the ability of the method to assess unequivocally the analyte in presence of components, which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc. [6]. The specificity of the developed LC method for zoledronic acid was carried out in the presence of its impurities namely Imp-1 and Imp-2.

Stress studies were performed for bulk drug to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to stress conditions of heat (60 °C), light, acid hydrolysis (0.5N HCl), base hydrolysis (0.1N NaOH), water hydrolysis and oxidation (10% H_2O_2 at room temperature and 10% H_2O_2 at 60 °C) to evaluate the ability of the proposed method to separate zoledronic acid from its impurities and degradation products. For thermal and light degradation, the study period was 10 days, whereas for acid, base, water hydrolysis and oxidation, it was 48 h. The photo degradation was carried out by exposing the zoledronic acid samples in solid state to light providing an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 W h/m^2, which took about 10 days period in our photo stability chamber. Stressed samples of zoledronic acid generated were checked for peak purity by using PDA detector. The peak purity of zoledronic acid was satisfactory in all stressed samples tested. Assay studies were carried out for stress samples against qualified reference standard and

the mass balance (%assay + %impurities + %degradation products) was calculated. Assay was also calculated for bulk sample by spiking two available impurities (Imp-1 and Imp-2) at the specification level (0.15%).

2.6. Method validation

2.6.1. Precision

The precision of the assay method was evaluated by carrying out six independent assays of test sample of zoledronic acid against qualified reference standard and calculated the % R.S.D. of assay.

The precision of the related substance method was checked by injecting six individual preparations of (1.0 mg/ml) zoledronic acid spiked with 0.15% of Imp-1 and Imp-2 with respect to analyte concentration. % R.S.D. of area for each Imp-1 and Imp-2 was calculated.

The intermediate precision of the method was also evaluated using different analyst and a different instrument in the same laboratory.

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

The LOD and LOQ for Imp-1 and Imp-2 were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentration [7]. Precision study was also carried at the LOQ level by injecting six individual preparations of Imp-1 and Imp-2 and calculating the % R.S.D. of the area.

2.6.3. Response function

Response function corresponds to the assessment of the relationship between the response (i.e. chromatographic signal) and the concentration (amount) of analyte in the sample system [8]. Test solutions for assay method were prepared from stock solution at six concentration levels from 25 to 150% of assay analyte concentration (50, 100, 150, 200, 250 and 300 μg/ml). The peak area (i.e. chromatographic signal) versus concentration (i.e. amount) data was performed by least-squares linear regression analysis.

Test solutions for related substance method were prepared by diluting the impurity stock solution (2.4) to the required concentrations. The solutions were prepared at six concentration levels from LOQ to 200% (i.e. 0.15%) of the specification (0.05, 0.075, 0.15, 0.1875, 0.225 and 0.3%). The calibration curve was drawn by plotting the peak areas of Imp-1 and Imp-2 versus its corresponding concentration.

Above tests were carried out for 3 consecutive days in the same concentration range for both assay and related substance method. The % R.S.D. value of the slope and *Y*-intercept of the calibration curve was calculated.

2.6.4. Accuracy

The accuracy of the assay method was evaluated in triplicate at three concentration levels, i.e. 100, 200 and 300 μg/ml in bulk drug sample. The percentage of recoveries were calculated from the slope and *Y*-intercept of the calibration curve obtained in Section 2.6.3. Accuracy/recovery experiments were performed in triplicate.

The bulk sample, provided by Process Research Department of Custom Pharmaceutical Services, does not show the presence of Imp-1 and Imp-2. Standard addition and recovery experiments were conducted to determine accuracy of the related substance method for the quantification of two impurities, namely Imp-1 and Imp-2, in bulk drug samples.

The study was carried out in triplicate at 0.075, 0.15 and 0.225% of the analyte concentration (1000 μg/ml). The percentage of recoveries for Imp-1 and Imp-2 were calculated from the slope and *Y*-intercept of the calibration curve obtained in Section 2.6.3.

2.6.5. Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution between zoledronic acid, Imp-1 and Imp-2 was recorded.

The flow rate of the mobile phase was 0.7 ml/min. To study the effect of flow rate on the resolution, it was changed by 0.1 units from 0.6 to 0.8 ml/min while the other mobile phase components were held constant as stated in Section 2.3. The effect of change in the composition of organic modifier was checked by changing the mobile phase composition to 96:4 (buffer:methanol) and also 94:6. The effect of column temperature on resolution was studied at 20 and 30 °C instead of 25 °C while the other mobile phase components were held constant as stated in Section 2.3.

2.6.6. Solution stability and mobile phase stability

The solution stability in the assay method was carried out by leaving both the test solutions of sample and reference standard in tightly capped volumetric flasks at room temperature for 48 h. The same sample solutions were assayed for every 6 h interval up to the study period. The mobile phase stability was also carried out by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions for 6 h interval up to 48 h. Mobile phase prepared was kept constant during the study period. The % R.S.D. of assay of zoledronic acid was calculated for the study period during mobile phase and solution stability experiments.

Leaving spiked sample solution in tightly capped volumetric flask at room temperature for 2 days carried out the solution stability of in the related substance method. Content of Imp-1 and Imp-2 were determined for every 6 h interval up to the study period. Mobile phase stability was also carried out for 2 days by injecting the freshly prepared sample solutions for 6 h interval. Contents of Imp-1 and Imp-2 were checked in the test solutions. Mobile phase prepared was kept constant during the study period.

3. Results and discussion

3.1. Method development and optimization

Imp-2 was the potential impurity expected to present in bulk samples produced by Dr. Reddy's Laboratories. The key objective of the chromatographic method is to get the separation of Imp-1 and Imp-2 from the analyte peak. Different stationary phases like C18, cyano, C8 and silica were evaluated for the separation of impurities, degradation products from zoledronic acid. In all possible trails using different make columns, early retentions were observed for zoledronic acid, its impurities and degradation products. The co-elution of impurities was observed, even after employing the use of ion-pairing agent. X Terra RP18, 250 mm × 4.6 mm, 5 μm HPLC column was found suitable to achieve the separation of impurities and degradation products from zoledronic acid drug substance. Even though X Terra RP18 column is specifically designed to avoid the use of ion-pairing agents, it has become necessary in case of zoledronic acid to achieve a good resolution from its impurities and degradation products. The column efficiency was found good even after making 500 injections. Impurities were also co-eluted by using different stationary phases like C18, C8 and cyano and different mobile phases containing buffers like phosphate, sulphate and citrate with different pH (4–7) and using organic modifiers like acetonitrile and methanol in the mobile phase. The concentration of the ion-pairing agent, tetra-*n*-butyl ammonium hydrogen sulphate and the percentage of organic modifier, methanol has played a significant role in achieving the separation between Imp-1, Imp-2 and zoledronic acid.

Satisfactory separation was achieved on a Waters X Terra RP18 (250 mm × 4.6 mm, 5 μm) column using a mixture of aqueous 8 mM di-potassium hydrogen ortho phosphate, 2 mM di-sodium hydrogen ortho phosphate and 7 mM tetra-*n*-butyl ammonium hydrogen sulphate (pH of mixture is about 6.6) and methanol (95:5, v/v). In the optimized chromatographic conditions, the resolution between zoledronic acid and its potential impurities namely Imp-1 (imidazole) and Imp-2 (imidazole-1-yl acetic acid) was found to be greater than 3. The typical retention times of Imp-1, Imp-2 and zoledronic acid were about 3.9, 4.8 and 6.8 min, respectively. The system suitability results are given in Table 1 and the developed LC method was found to be specific for zoledronic acid, its two impurities namely Imp-1, Imp-2 and its degradation products.

Table 1
System suitability report

Compound ($n=3$)	USP resolution	USP tailing factor (R_S)	No. of theoretical plates (N), USP tangent method
Imp-1	–	1.8	4986
Imp-2	3.88	1.3	9360
Zoledronic acid	6.22	1.9	3668

n, Number of determinations.

3.2. Method validation

3.2.1. Precision

The % R.S.D. of assay of zoledronic acid during assay method precision study was within 1% and the % R.S.D. of area of Imp-1 and Imp-2 in related substance method precision study was within 8%. The % R.S.D. of assay results obtained in intermediate precision study was within 0.9% and the % R.S.D. of area of Imp-1 and Imp-2 were well within 10%, confirming good precision of the method.

3.2.2. Limit of detection and limit of quantification

The limit of detection of both Imp-1 and Imp-2 was achieved at 0.02% (of analyte concentration, i.e. 1000 μg/ml) for 10 μl injection volume. The limit of quantification of both Imp-1 and Imp-2 was achieved at 0.05% (of analyte concentration, i.e. 1000 μg/ml) for 10 μl injection volume. The method precision for Imp-1 and Imp-2 at LOQ level was below 9%. The accuracy/recovery experiments were carried out for both Imp-1 and Imp-2 at LOQ concentration in triplicate and the recoveries were found to be well within 85–115%, which demonstrate that the developed method can accurately measure the impurities present in zoledronic acid.

3.2.3. Response function

Calibration plot for assay method was obtained over the calibration ranges tested, i.e. 50–300 μg/ml and the correlation coefficient obtained was greater than 0.999. Chromatographic response was checked for assay method over the same concentration range for 3 consecutive days. The % R.S.D. values of the slope and *Y*-intercept of the calibration curves were 3.1 and 5.5, respectively. The results show that a good correlation existed between the peak area and concentration of the analyte.

Calibration plot for related substance method was obtained over the calibration ranges tested, i.e. LOQ (0.05%) to 0.3% (200% of specification level, i.e. 0.15%) for Imp-1 and Imp-2. The correlation coefficient obtained was greater than 0.990. Chromatographic response was checked for related substance method over the same concentration range for 3 consecutive days. The % R.S.D. values of the slope and *Y*-intercept of the calibration curves were 3.9 and 10.5, respectively. The above results show that an excellent correlation existed between the response and concentration of Imp-1 and Imp-2 (Fig. 2).

3.2.4. Accuracy

The percentage recovery of zoledronic acid in bulk drug samples was ranged from 99.2 to 100.8 (Table 2). The percentage recovery of Imp-1 and Imp-2 in bulk drugs samples was ranged from 91.5 to 104.5. HPLC chromatograms of blank, pure (unspiked) and spiked samples at 0.15% level of all two impurities in zoledronic acid bulk drug sample are shown in Fig. 3.

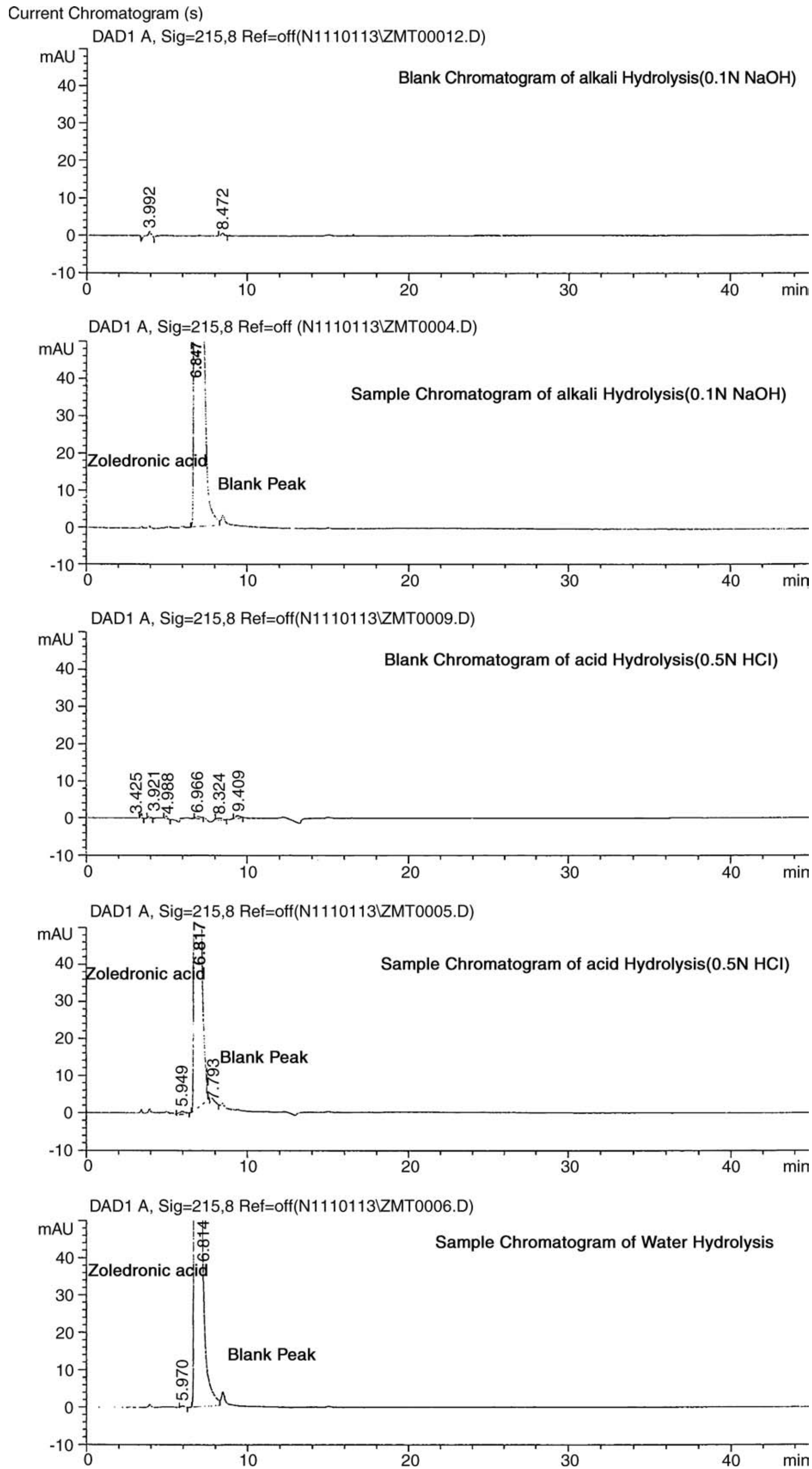


Fig. 2. Typical HPLC chromatograms related to stress studies.

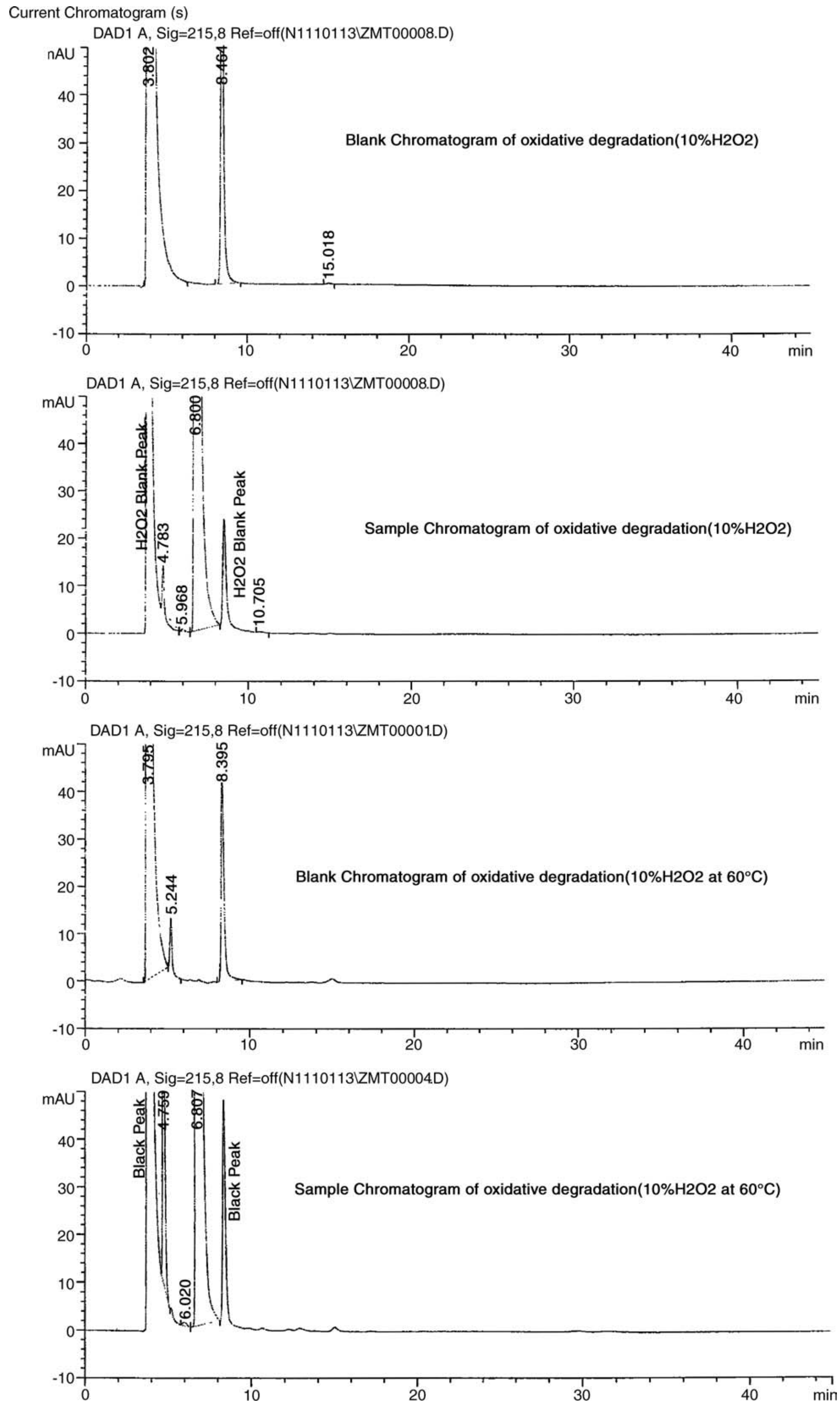


Fig. 2. (*Continued*).

Table 2
Recovery results of in bulk drug sample

Added (μg) ($n = 3$)	Recovered (μg)	Percentage of recovery	% R.S.D.
101	100.7	99.7	0.8
200	198.4	99.2	0.6
302	304.4	100.8	1.0

$n = 3$ determinations.

3.2.5. Robustness

In all the deliberate varied chromatographic conditions (flow rate, composition of organic modifier and column temperature), the resolution between critical pair, i.e. Imp-2 and zoledronic acid was greater than 3, illustrating the robustness of the method (Table 3).

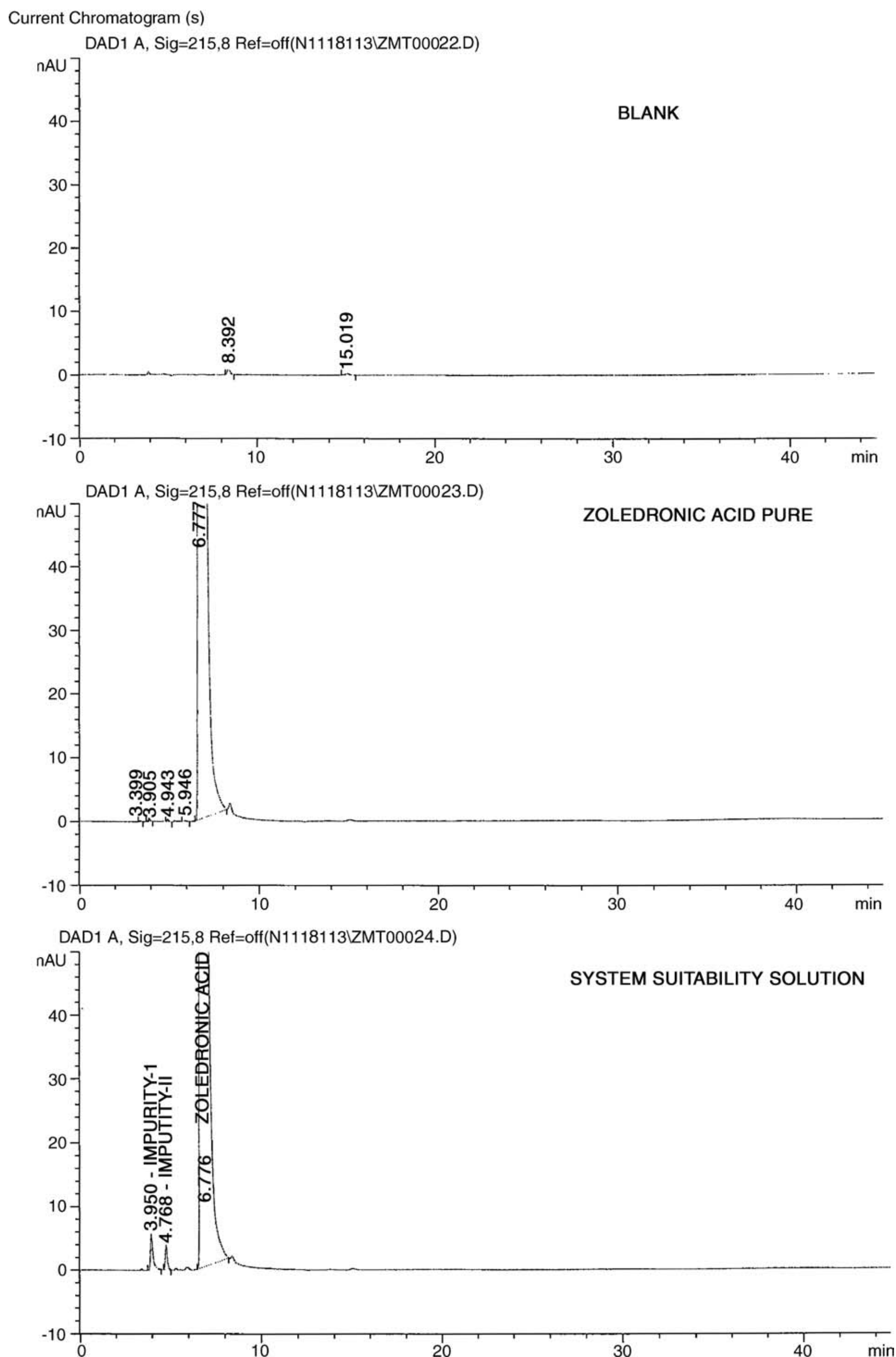
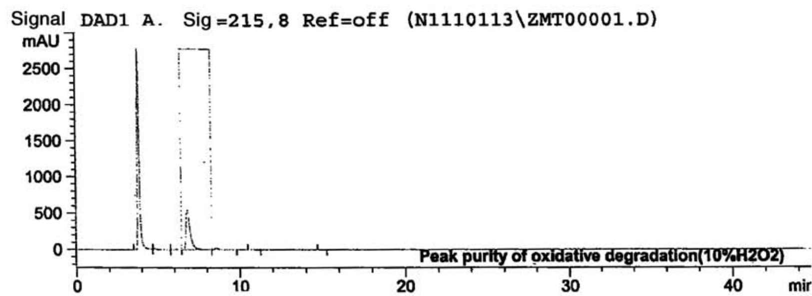
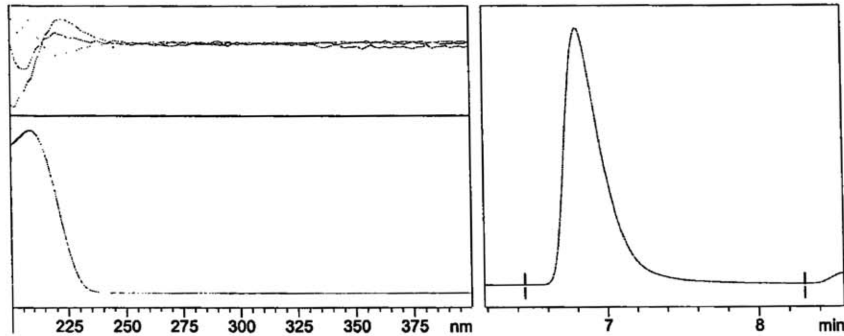


Fig. 3. HPLC chromatograms of blank, unspiked (pure bulk) and spiked samples.

Purity results peak 4 at 6.800 min.

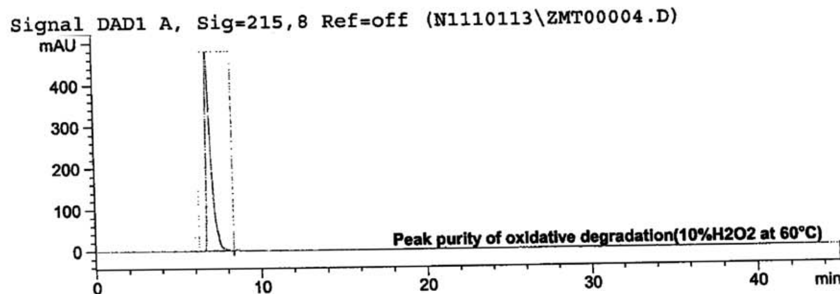


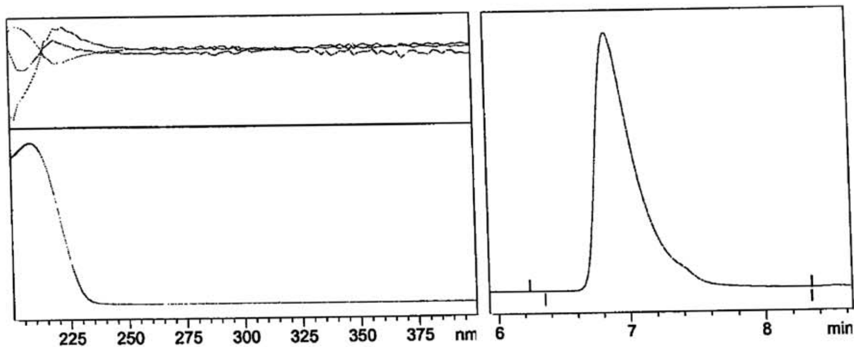


-> The purity factor is within the threshold limit. <-

```
Purity factor : 999.995 (3 of 3 spectra are within the threshold limit.)
Threshold     : 990.000  (Set by user)
Reference     : Peak start and end spectra (integrated) (6.446 / 8.300)
Spectra       : 3  (Selection automatic, 3)
```

Purity results peak 1 at 6.847 min.





-> The purity factor is within the threshold limit. <-

```
Purity factor : 999.995 (3 of 3 spectra are within the threshold limit.)
Threshold     : 990.000  (Set by user)
Reference     : Peak start and end spectra (integrated) (6.350 / 8.337)
```

Fig. 4. Typical HPLC chromatograms demonstrating peak purity.

Table 3
Results of robustness study

S. no.	Parameter	Variation	Resolution between Imp-2 and zoledronic acid
1.	Temperature (±5 °C of set temperature)	(a) At 20 °C	5.1
		(b) At 30 °C	4.9
2.	Flow rate (±20% of the set flow)	(a) At 0.55 ml/min	5.3
		(b) At 0.85 ml/min	4.7
3.	Mobile phase composition (±10% of organic modifier)	(a) At 4.5 ml	5.7
		(b) At 5.5 ml	4.6

Table 4
Summary of forced degradation results

Stress condition	Time	%Assay of active substance	Mass balance (%assay + %impurities + %degradation products)	Remarks
Acid hydrolysis (0.5N HCl)	48 h	99.3	99.8	No degradation products formed
Base hydrolysis (0.1N NaOH)	48 h	99.5	100.1	No degradation products formed
Oxidation (10% H_2O_2 at 60 °C)	48 h	90.0	99.6	Imp-2 and some unknown degradation products formed
Thermal (60 °C)	10 days	99.8	99.9	No degradation products formed
Light	10 days	99.6	99.8	No degradation products formed
Water hydrolysis	48 h	99.9	99.8	No degradation products formed
Oxidation (10% H_2O_2 at room temperature)	48 h	99.5	99.7	Mild degradation observed

3.2.6. Solution stability and mobile phase stability

The solution stability and mobile phase stability experiments were performed as described in Section 2.6.6. The % R.S.D. of assay of zoledronic acid during solution stability and mobile phase stability experiments were within 1.0% R.S.D. No significant change was noticed in the content of Imp-1 and Imp-2 during solution stability and mobile phase stability experiments. The data obtained in both the above experiments proves that sample solutions and mobile phase used during assay and related substance determination were stable up to 48 h.

3.3. Results of forced degradation studies

Degradation was not observed in zoledronic acid samples when subjected to stress conditions like light, heat, acid, alkali and water hydrolysis (Fig. 2). Mild degradation was observed when the drug substance was treated with 10% hydrogen peroxide at room temperature. The considerable degradation of drug substance was observed only in oxidative conditions when zoledronic acid was treated with 10% hydrogen peroxide at 60 °C (Fig. 2). Zoledronic acid was degraded to Imp-2 during oxidative degradation (in 10% hydrogen peroxide at 60 °C after 48 h treatment) and it was confirmed by co-injection with a qualified Imp-2 standard. The drug substance zoledronic acid under oxidative conditions (in 10% hydrogen peroxide at 60 °C after 48 h treatment) also leads to the formation some unknown degradation peaks. Peak purity test results confirm that the zoledronic acid peak is homogeneous and pure in all the analyzed stress samples of zoledronic acid (Fig. 4). The stressed samples were assayed against a qualified reference standard of zoledronic acid. The mass balance (%assay + %impurities + %degradation products) in all stressed samples tested was close to 100% (Table 4). The assay of zoledronic acid is unaffected in the presence of its impurities namely Imp-1 and Imp-2 and also in presence of unknown degradation products which confirms the stability indicating power of the method.

4. Conclusions

The isocratic ion-pair RP-LC method developed for the determination of related substances and assay of zoledronic acid drug substance is precise, accurate and selective. The method was fully validated showing satisfactory data for all the method validation parameters tested. The developed method is a stability indicating and can be conveniently used by quality control department to determine the related substances and assay in regular zoledronic acid production samples and also stability samples.

Acknowledgements

The authors wish to thank the management of Dr. Reddy's Group for supporting this work. Authors wish to acknowledge the Process Research Group for providing the samples for our research. We would also like to thank colleagues in separation science division of Analytical Research of Custom Pharmaceutical Services for their co-operation in carrying out this work.

References

[1] S. Singh, M. Bakshi, Pharm. Technol. Online 24 (2000) 1–14.
[2] M. Bakshi, B. Singh, A. Singh, S. Singh, J. Pharm. Biomed. Anal. 26 (2001) 1011–1040.
[3] M. Bakshi, S. Singh, J. Pharm. Biomed. Anal. 28 (2002) 891–897.
[4] Y. Jiang, X.Q. Zhang, Z.R. Xu, Chromatographia 60 (2004) 405–409.
[5] F. Legay, S. Gauron, F. Deckert, G. Gosset, U. Pfaar, C. Ravera, H. Wiegand, H. Schran, J. Pharm. Biomed. Anal. 30 (2002) 897–911.
[6] ICH, Text on Validation of Analytical Procedures, Q2A.
[7] ICH, Validation of Analytical Procedures, Methodology, Q2B.
[8] Ph. Hubert, J. Pharm. Biomed. Anal. 36 (2004) 579–586.