

Sensitive and Rapid RP-HPLC Quantification of Zoledronic Acid in a Hydroxyapatite-based Nanoparticles

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Khajuria and Razdan: RP-HPLC Determination of Zoledronic Acid in Nanoparticles

The aim of this study was to develop and validate a rapid and sensitive reverse-phase high performance liquid chromatography method for the quantitative determination of zoledronic acid in nanoparticles. The mobile phase was a mixture of methanol (30%) and 7 mM tetra butyl ammonium hydrogen sulphate, 2 mM di-sodium hydrogen orthophosphate and sulphate and 8 mM di-potassium hydrogen orthophosphate (70%), pH adjusted to 3 with trifluoroacetic acid. C18 column was used as a stationary phase. The flow rate was maintained at 1 ml/min. The detection of the constituents was done at 215 nm using an ultraviolet detector. Method validation was executed according to the International Conference on Harmonisation guidelines. The retention time of zoledronic acid was 3.27 ± 0.03 min (n=6). The assay was linear over the concentration range of 200-800 µg/ml. The proposed method was sensitive with the limit of detection and limit of quantification values equal to 200 and 800 µg/ml, respectively. The method was accurate with percent mean recovery from 99.01 ± 1.31 to $100.80 \pm 0.094\%$ and precise as percent relative standard deviation values were less than 1%. The method was successfully applied for the estimation of zoledronic acid loading efficiency on hydroxyapatite nanoparticles and *in vitro* zoledronic acid release profile analysis in hydroxyapatite-based nanoparticles.

Key words: Zoledronic acid, nanomedicine, RP-HPLC, validation, ICH guidelines

Bisphosphonates are one of the most important bone resorption inhibitor drugs, which are used extensively in the management of skeletal diseases like osteoporosis, malignant hypercalcemia, bone metastasis and Paget's disease^[1-3]. Bisphosphonates are the endogenous arbitrator of bone mineralization, capable of being adsorbed onto hydroxyapatite (HA) crystals in bone, hence providing bone stabilization and hindering its dissolution. Unlike pyrophosphate (P-O-P), bisphosphonates due to their P-C-P structure are more stable and resistant to acid hydrolysis^[4]. Despite their therapeutic effects, bisphosphonates are currently used in various studies as suitable targeting agents in radiotherapy^[4,5]. Previous studies from our laboratory and others have shown that bisphosphonates could be useful as drug bone targeted carriers for delivering various nanoparticle-based therapeutic interventions to bone tissues for skeletal-related diseases^[6-10]. This approach prevents adverse effects such as toxicity due to non-specific tissue interactions, larger medicine dosages, higher costs, and even likely poor patient compliance towards therapy.

Zoledronic acid (ZOL) is a newer third generation bisphosphonate. It is a potent inhibitor of osteoclastic bone resorption. ZOL chemically is [1-hydroxy-2-(1*H*-imidazol-1-yl)ethane-1,1-diyl]bis(phosphonic acid) hydrate (fig. 1)^[1-3]. Among bisphosphonates, ZOL has a higher binding affinity for the bone HA. It was established that under conditions likely to stimulate bisphosphonate binding to the bone, ZOL has a superior binding affinity for bone HA crystals as compared to other bisphosphonates. Therefore, after intravenous injection, ZOL is swiftly cleared from the circulation and gets localized to the bone^[7].

Literature survey revealed several reverse phase-high performance liquid chromatography (RP-HPLC)

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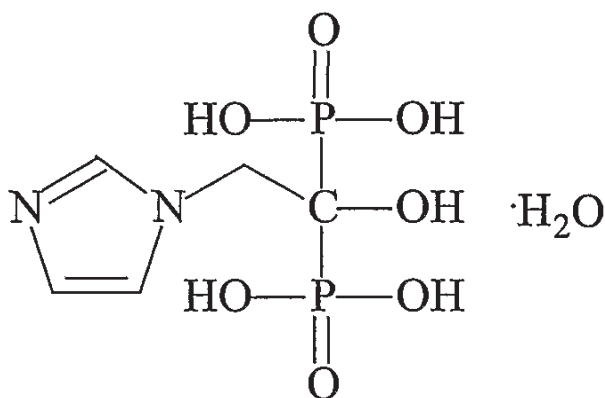


Fig. 1: Chemical structure of zoledronic acid

methods for determination of ZOL in injections and tablets^[11-14]. But most of these methods are tedious, time-consuming, and require complex sample preparations. However, a simple and rapid quantitative HPLC method for estimation of ZOL in nanoparticle-based formulations has not yet been reported. Therefore, development of a suitable RP-HPLC analytical method for estimating ZOL in bone-targeted nanocarrier-based formulations appeared to be essential.

Development of a chromatographic method for the quantitative estimation of ZOL in nanoparticle-based formulations presented the following major difficulties, ZOL can chelate to metals and metal surfaces, generating chromatographic peak tailing; ZOL has no ultraviolet absorption or fluorescence, hence not readily detectable and as ZOL consisted of two phosphonate groups connected to a single carbon atom, it is poorly retained on non-polar stationary phase such as C₁₈ or C₈ column. For these reasons, ion-exchange liquid chromatographic methods are required for analysis of ZOL^[11]. The main objective of this study was to develop a sensitive and rapid RP-HPLC method to assay ZOL in HA-based nano formulation for osteoporosis. The retention problem of ZOL has been successfully resolved by adding ion-pair reagent in the mobile phase. Our study appears to be the first to report a short run time of 3.2 min in the RP-HPLC method for ZOL, which facilitated analysis of several hundred samples per day. This RP-HPLC method offered significant advantages owing to its simplicity and rapidness for the detection of ZOL in the nanoparticle-based formulations.

MATERIALS AND METHODS

ZOL was obtained from Naprod Life Sciences Pvt. Ltd., Thane, Maharashtra, India. HPLC grade calcium nitrate tetrahydrate, phosphoric pentoxide,

ethanol, sodium hydroxide, methanol, tetra butyl ammonium hydrogen sulphate, di-potassium hydrogen orthophosphate, trifluoroacetic acid and di-sodium hydrogen orthophosphate were purchased from Merck, Mumbai, India. High pure water was prepared by using Millipore Milli Q plus purification system.

Preparation of bone targeted ZOL-loaded HA nanoparticles:

ZOL-loaded HA nanoparticles were prepared according to our previously reported method^[7]. Briefly, HA nanoparticles were synthesized first by sol-gel method in which calcium nitrate tetrahydrate and phosphoric pentoxide were used with the molar ratio of 10:3. Absolute ethanol was used as the solvent. After dissolving, the above precursors slowly transformed into a gel after 24 h under continuous stirring at ambient temperature. The formed gel was dried in an oven at 80° in the air for 24 h and then sintered at 600°. Adsorption method was employed for the preparation of ZOL-loaded HA nanoparticle-based formulation^[7].

For estimation of ZOL loading on HA, 10 mg weighed sample of dried nanoparticles was dissolved in 5 ml of methanol. The sample was sonicated for 20 min, made up to 10 ml with methanol, and was filtered using a 0.2 µm syringe filter (Axiva Sicheem Biotech, New Delhi, India). ZOL content in the formulation was determined by RP-HPLC and drug loading efficiency was calculated^[15]. For *in vitro* release studies, about 100 mg of HA/ZOL nanoparticle-based drug formulation was transferred to a 100 ml beaker containing 50 ml of phosphate buffer saline (PBS) pH of 7.4 and continuously stirred at 100 rpm 37°. Samples were withdrawn at various time intervals from the beaker and ZOL content was measured using the RP-HPLC method. An equal volume of PBS replaced the samples that were withdrawn.

HPLC instrumentation and chromatographic conditions:

The HPLC system consisted of a Shimadzu LC-20AD precision isocratic pump and an SPD-20A UV/Vis detector (Kyoto, Japan). Data collection and integration was accomplished utilizing LC Solutions chromatography Data System (Kyoto, Japan). A PHS-2C precision pH meter (Elico, LI 614, Hyderabad, India) equipped with a combination pH electrode was used for pH determination of the mobile phase. The chromatographic separation was achieved on a Phenomenex Luna C18 (2) analytical

column (250×4.6 mm, 5 µm particle size) fitted with a Phenomenex analytical security Guard Cartridge System. The mobile phase consisted of a mixture of phosphate buffer that contained, 7 tetra butyl ammonium hydrogen sulphate, 2 mM di-sodium hydrogen orthophosphate and sulphate and 8 mM di-potassium hydrogen orthophosphate (pH of the mixture was adjusted to 3 with trifluoroacetic acid) and methanol (70:30, v/v). The composition of the phosphate buffer was prepared according to the ZOL HPLC assay method previously reported by Rao *et al.*^[13]. The flow rate of the mobile phase was 1 ml/min. The column temperature was maintained at 25° and the wavelength was monitored at 215 nm. The injection volume was 20 µl. The total run time for each LC run for the assay estimation was 8 min.

Linearity and range:

A stock solution of ZOL (10 mg/ml) was prepared in methanol. The solutions were filtered through a 0.45 µm nylon filter. Successive volumes of the standard solution were injected and chromatograms were recorded until the reproducibility of the peak area was satisfactory. Several standard solutions in the range of 200-800 µg/ml were used to define linear range of ZOL. The data of peak area versus drug concentration were treated by linear least square regression.

Accuracy as recovery:

Accuracy was determined by recovery study using standard addition method. The pre-analysed samples were spiked with extra 50, 100, and 150% of the standard ZOL and the mixtures were analysed by the proposed method. The experiment was conducted in triplicate.

Precision:

The intraday (repeatability) and interday (intermediate precision) variations for the determination of ZOL was carried out at three concentration levels of 200, 400, and 800 µg×ml⁻¹. The determinations were carried out in triplicate.

Robustness:

Robustness was carried out by making very small changes in the analytical methodology at a single concentration level (400 µg×ml⁻¹). Robustness was determined by making deliberate modifications in the mobile phase ratio, flow rate, and detection wavelength for analysis. The percentage of relative standard deviation (%RSD) was also calculated to assess the

robustness of the proposed method.

Detection and quantification limits:

The limit of detection (LOD) is the minimum amount of analyte in a specimen, which can be identified but not necessarily quantitated. The limit of quantification (LOQ) is the minimum amount of analyte in a specimen, which can be quantitatively determined with proper precision and accuracy. The LOD and LOQ were determined based on the method of signal-to-noise ratio (International Conference on Harmonization guidelines ICH-Q2 (R1) 2005) utilizing the following Eqns., $LOQ=10 \sigma/S$, $LOD=3.3 \sigma/S$, where, σ is the standard deviation of the intercept of the calibration plot and S is the slope of the calibration curve.

RESULTS AND DISCUSSION

Although limited RP-HPLC methods for the determination of ZOL have been reported^[11-14], none of them reported their specificity in quantifying ZOL in nanostructured delivery systems. No methods are available for ZOL quantification from nanoparticle-based formulations where a high specificity is required to overcome the probable interference of the excipients. The primary goal was to develop a sensitive and rapid RP-HPLC method that can assay ZOL. The sensitivity of RP-HPLC method that uses UV-detection depends on upon the proper selection of wavelength. An injection of 400 µg/ml of the standard solution of ZOL was made and the UV/Vis profile was recorded (200-400 nm). From the UV/Vis profile, a detection wavelength of 215 nm was selected, as the drug gave better peak response. The detection wavelength of 215 nm was selected and satisfactory precision was achieved. In this wavelength, ZOL could be determined with high sensitivity.

Satisfactory assay method of ZOL was achieved with a Phenomenex Luna C18 (2) analytical column (250×4.6 mm, 5 µm particle size) using a mixture of aqueous, 7 tetra butyl ammonium hydrogen sulphate, 2 mM di-sodium hydrogen orthophosphate and sulphate and 8 mM di-potassium hydrogen orthophosphate (pH 3) and methanol (70:30, v/v). The column efficiency was found good even after making 500 injections. Optimization of chromatographic conditions, which is based on reasonable retention time for ZOL and excellent peak shape of ZOL was achieved initially. ZOL has multiple pKa values due to the presence of the phosphate moiety attached to the single carbon atom. In IEC, selection of most appropriate buffered

mobile phase (as counter ion), its concentration and pH would significantly affect the extent of ionization of compounds and, hence, their retention times. For this purpose, the buffer concentration was kept as low as possible to avoid competition of the counter ions with the retention of sample ions. The addition of an organic solvent to the mobile phase resulted in the decreased retention just as in case of reversed-phase chromatography. Also, tetra butyl ammonium hydrogen sulphate was utilized as the ion-pair reagent. The optimized method developed resulted in the elution of ZOL at 3.27 ± 0.03 . Fig. 2 showed the chromatogram of pure ZOL.

The ZOL-HA nanoparticle solution did not show any changes in the peak at the retention time of pure ZOL. This indicates that the excipients used in the nanomedicine product do not interfere in estimation ZOL. Hence, the specificity for ZOL peak under the selected chromatographic conditions was proved. The ZOL peak identity was proved by comparing

the retention times of pure ZOL with the HA-ZOL nanomedicine, as shown in fig. 2.

Several standard solutions of ZOL were used to define a linear range. The calibration curve was linear in the range of 200-800 $\mu\text{g/ml}$. A good linearity was established by a correlation coefficient (R^2) value of 0.9994 ± 0.0001 (Table 1). A correlation coefficient is a statistical parameter used to measure the degree or strength of the type of relationship, and here, a higher correlation coefficient value (close to 1.0) indicated a high level of linear relationship between the concentration of ZOL and the peak area. No significant differences were observed in the slopes of standard curves as indicated by the low %RSD of 0.30. Table 2 shows the linear regression data for the calibration curve of ZOL.

Accuracy was examined by evaluating three concentrations of the standard ZOL solution previously analysed using standard addition technique. The

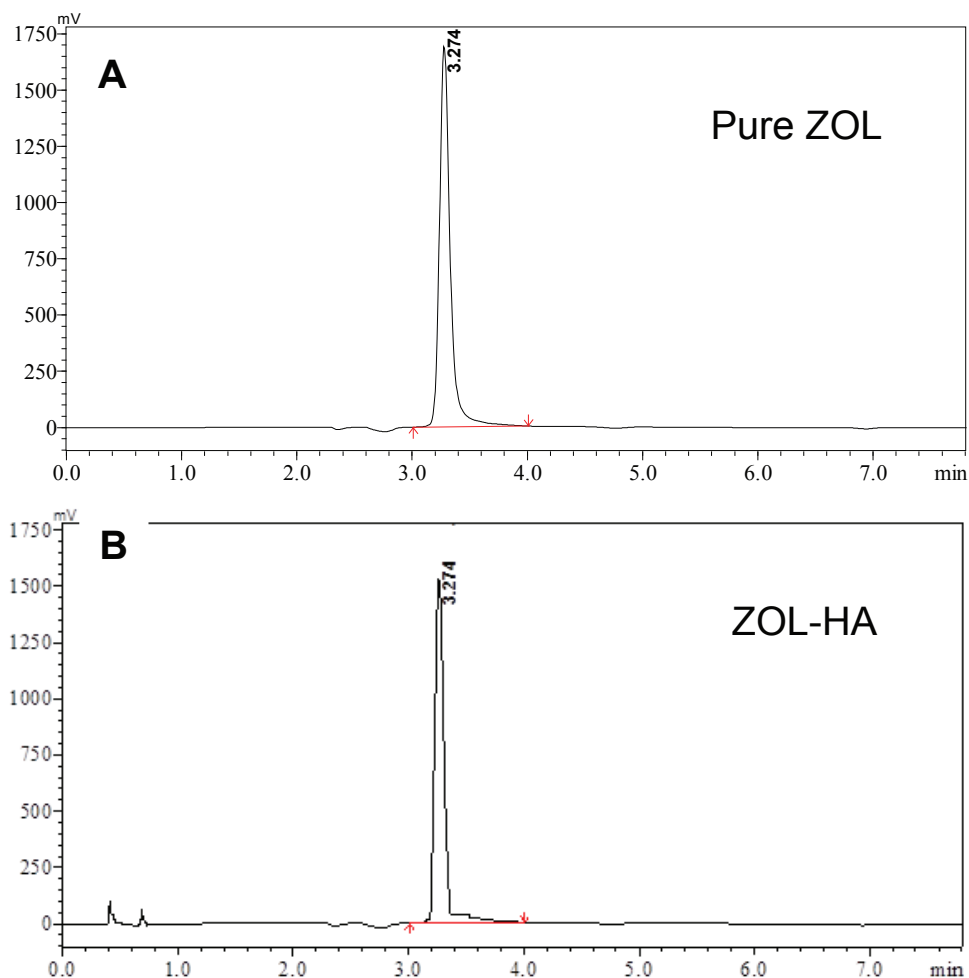


Fig. 2: Typical chromatogram ZOL and ZOL-HA nanoparticles
Chromatograms of A. pure zoledronic acid (ZOL) and B. zoledronic acid-hydroxyapatite (ZOL-HA) nanoparticles

recovery study was carried out to evaluate the accuracy of the proposed method to estimate ZOL. The standard addition technique was performed by adding 50, 100, and 150% of ZOL concentration in the sample. The percentage recoveries of the three ZOL concentrations were found to be 99.01 ± 1.31 to 100.80 ± 0.094 , which indicated high accuracy. Percentage recovery and %RSD values are shown in Table 2. The mean percentage recovery values, close to 100%, and their low %RSD values indicated a high accuracy of the analytical method.

The repeatability of the developed RP-HPLC method, by intraday assay, was expressed in the terms of %RSD, and the results (Table 3) demonstrated the repeatability of the method. The inter day variation of ZOL at three different concentration levels of 200, 400, and $800 \mu\text{g ml}^{-1}$ establishes the intermediate precision of the

method. The low values of %RSD for repeatability and intermediate precision shows an excellent precision of the developed RP-HPLC method.

Robustness was investigated by introducing minor changes in the mobile phase ratio, flow rate, and detection wavelength for analysis. The standard deviation and %RSD of peak area and retention time (R_t) was calculated and shown in Table 4. The low values of %RSD show the robustness of the developed RP-HPLC method. The LOD and LOQ were determined as per the ICH Guidelines Q2 (R1) (2005) and were found to be 200 and $800 \mu\text{g} \times \text{ml}^{-1}$, respectively.

Fig. 3 shows the *in vitro* release of ZOL from HA/ZOL nanoparticles, which ranged between $85.28 \pm 4.86\%$ and $69.82 \pm 4.84\%$ depending on the HA-to-ZOL ratio used. HA-to-ZOL ratio of 0.75:1 showed the maximum *in vitro* release of $85.28 \pm 4.86\%$ after 70 min of dissolution in PBS.

A sensitive and rapid RP-HPLC method was developed and validated for the quantitative analysis of ZOL in nanoparticle-based formulations. The method validation was performed according to the ICH guidelines. The developed method was demonstrated

TABLE 1: LINEAR REGRESSION DATA FOR THE CALIBRATION CURVE

Parameters	Mean \pm SD	%RSD
Linearity range ($\mu\text{g/ml}$)	200 to 800	-
Correlation coefficient (R^2)	0.9994 ± 0.0001	0.02
Slope	13619.24 ± 13.98	0.30
Intercept	10906.51 ± 825.62	1.02

(n=3)

TABLE 2: RECOVERY DATA FOR THE ACCURACY OF THE HPLC METHOD

Excess ZOL added (%)	Content of sample ($\mu\text{g/ml}$)	Theoretical content of spiked sample ($\mu\text{g/ml}$)	Estimated Content \pm SD ($\mu\text{g/ml}$)	Recovery \pm SD (%)	%RSD
50	400	450	451.32 ± 1.53	99.01 ± 1.31	1.15
100	400	500	502.72 ± 1.26	101.09 ± 0.78	0.68
150	400	550	551.29 ± 1.83	100.80 ± 0.94	0.71

TABLE 3: REPEATABILITY AND INTERMEDIATE PRECISION OF HPLC METHOD

Concentration of sample ($\mu\text{g/ml}$)	Mean peak area \pm SD	%RSD*	Mean peak area \pm SD	%RSD*
200	467291.0 ± 4415.3	0.62	467149.3 ± 3698.1	0.61
400	934586.5 ± 5215.7	0.41	934299.8 ± 4287.9	0.37
800	1869166.2 ± 6423.5	0.34	1868596.6 ± 7423.5	0.32

*Each value is a mean of three observations

TABLE 4: ROBUSTNESS DATA OF THE DEVELOPED RP-HPLC METHOD

Parameter	Study condition		Mean area \pm SD	%RSD* of area	$R_t \pm$ SD	%RSD* of R_t
	Original	Used				
Mobile phase ratio (Buffer/methanol)	70:30:00	68:32:00	934178.2 ± 4117.7	0.41	3.279 ± 0.031	0.45
		70:30:00				
		72:28:00				
Flow rate (ml/min)	1	0.9	934351.9 ± 5824.2	0.33	3.277 ± 0.078	0.42
		1				
		1.1				
Detection wavelength (nm)	215	213	934566.7 ± 3859.9	0.3	3.275 ± 0.024	0.3
		215				
		217				

*Each value is a mean of three observations

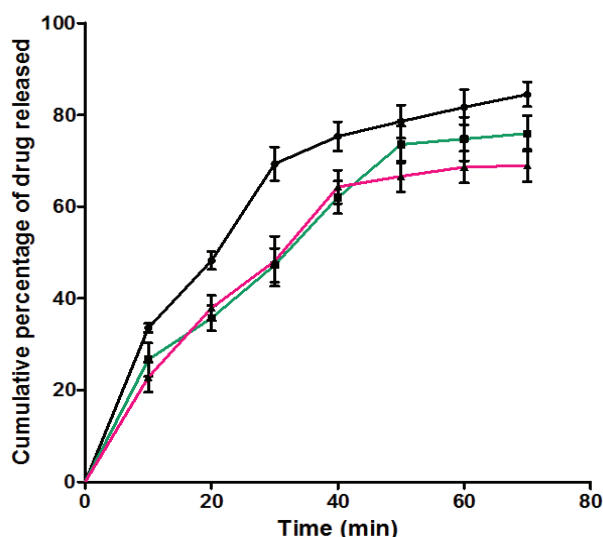


Fig. 3: Percent *in vitro* release of ZOL from HA/ZOL nanoparticles

Mean \pm SD, $n = 3$, —●— HA-ZOL (0.75:1), —■— HA-ZOL (0.50:1), —▲— HA-ZOL (0.25:1)

to be sensitive, rapid, accurate, and precise. The developed method could be successfully applied for the determination of the percentage ZOL loading, *in vitro* ZOL release profile and purification analysis of ZOL in nanoparticle-based drug formulations.

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Conflict of interest:

The authors declare no conflict of interest.

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