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Quantitative Determination of Alendronate in Human Urine

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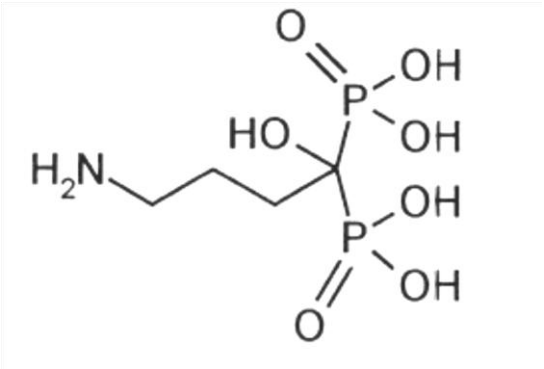
A rapid method based on high-performance liquid chromatography/electrospray–mass spectrometry (HPLC/ESI-MS) for the quantitative determination of alendronate in human urine has been developed and validated. Improved chromatographic separation and increased sensitivity of the detection was achieved by derivatisation. Higher efficiency of derivatisation as well as, more discriminatory recovery of the drug's derivatives was obtained by the use of 'on-cartridge' reaction with diazomethane. Important parameters such as sensitivity, linearity, matrix effect, reproducibility, stability, carry-over and recovery were investigated during the validation. The lower limit of detection was found to be 0.250 ng/mL. The intra- and inter-run precision, calculated from quality control (QC) samples was less than 5.0 %. The accuracy as determined from QC samples was in the range of 93.4–107.0% for the analyte. The mean recoveries for the low, medium and high quality control samples were 97.6 %, 97.0 % and 98.7 % respectively. Various conditions arising from potential interference peaks as a result of chromatographic separation of desired analytes were optimized. The developed method can provide a very useful technique for the analysis of drugs in human subjects.

Keywords: Alendronate; Liquid Chromatography-Mass Spectrometry/Mass Spectrometry; Derivatisation; Diazomethane; Human Urine; Bisphosphonates

1. INTRODUCTION

Alendronate, like other bisphosphonates, is a bone resorption inhibitor [1] being used in prevention and treatment of bone diseases. It is used in the prevention and treatment of postmenopausal osteoporosis [2], Paget's disease, primary hyperparathyroidism, malignant hypercalcemia and metastatic bone diseases. The pharmacological action of alendronate relies on its interfering with the mevalonate pathway by inhibiting farnesyl pyrophosphate(FPP) synthase [3], and thus reducing levels of geranylgeranyl diphosphate (GGPP), which is required for the prenylation of

guanosine triphosphate (GTP)-binding proteins (eg. Rab, Rac, Ras, Rho and Cdc42) that are essential for osteoclast activity and survival [4,5]. Consequently alendronate interferes with the stability of the ruffled border and stimulates osteoclast apoptosis, which reduces bone resorption, lowers bone turnover, and promotes a positive bone balance [6]. Moreover, studies indicate that bisphosphonates also influence osteoblasts and increase bone formation, and more recently others have reported that bisphosphonates enhance osteoblast proliferation and maturation [7-9], and inhibit osteoblast apoptosis [10]. Chemically alendronate is (4-amino-1-hydroxy-1-phosphonobutyl) phosphonic acid [A].



Scheme A. Chemical structure of alendronate

Analysis of alendronate in biological samples presents challenges in both sample purification and analyte detection. However, a survey of the literature revealed that few methods have been reported for its determination, such as several HPLC derivatisation methods using either pre-column [11-14] or post-column derivatisation [15,16] have been reported for its determination. Direct HPLC analysis of alendronate using refractive index detector [17], ion chromatography with conductivity detection [18] or indirect UV detection [19] have also been reported. Ion chromatography with mass spectrometry techniques have employed in characterization of alendronate sodium [20] and it was determined in pharmaceutical dosage forms by HPLC after derivatisation with 9-fluorenyl methylchloroformate (FMOC) [21]. Methods like inductivity coupled plasma and anodic stripping voltammetry have also been reported for the analysis of alendronate sodium in tablets [22,23]. Further, HPLC method with diode array detection (HPLC-DAD) and with fluorescence detection (HPLC-FD) has been developed for the determination of alendronate. Both methods depend on the ability of alendronate sodium to react with o-phthalaldehyde (OPA) at basic pH to produce light absorbing derivative [24]. The reported methods for quantitation of alendronate are all expensive and time consuming. In the last decades modern electrochemical techniques [25- 94] have also been widely used for the determination of the pharmaceuticals. The proposed method in this study has advantages of minimum sample pretreatment, faster extraction prior to the analysis, and cheaper reagents and equipment which makes the method of choice for routine pharmaceutical analysis.

The limit of detection for the determination of alendronate in urine by automated pre-column (RP-HPLC) derivatisation with 2,3-naphthalene dicarboxyaldehyde was found to be 2.5 and 1.0 ng/mL with electrochemical or fluorescent detector respectively [13]. However the total run time was significantly long (30 min). When FMOC was used to derivatize alendronate, a LOQ of 3.5 $ngml^{-1}$ was

achieved with fluorescence detection [14]. In that method a gradient system was required and total run time for one sample was 21 min. Immunochemical methods [95, 96] on the other hand offers higher sensitivity, but inadequate selectivity and matrix effect are some of the known inherent drawbacks of such methods. Thus, clinical analysis of alendronate demands a more sensitive and robust method.

Bisphosphonates were reported as not being amenable to analysis by HPLC with tandem mass spectrometric detection (HPLC/MS/MS) due to strong interaction with metal ions and their tendency to form adducts and multiple charged species [97]. The objective of the current work is to develop and validate the LC-MS/MS bioanalytical method which has high sensitivity, selectivity, minimizes matrix effect and has high sample throughput. The basis of the approach behind the objective is to derivatise alendronate with diazomethane [98] on silica-based anion exchange columns during solid-phase extraction (SPE).

2. MATERIALS AND METHODS

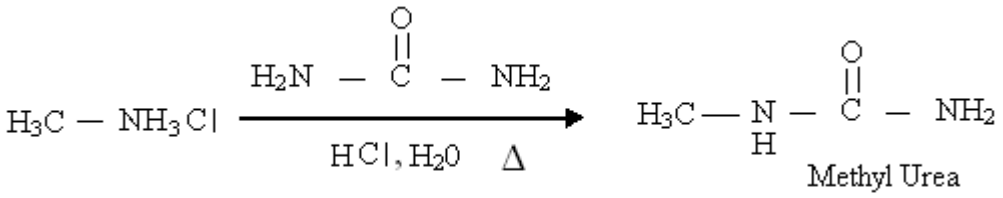
2.1. Reagents and materials

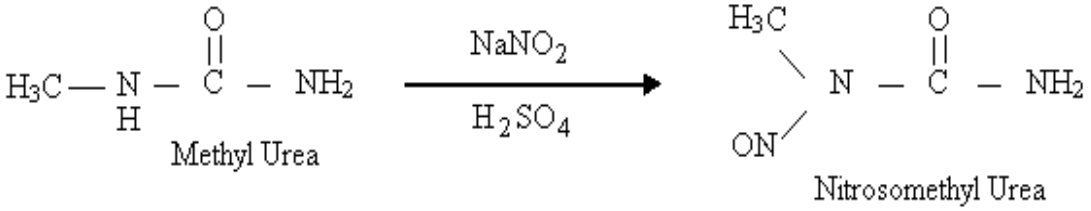
Alendronate sodium and d6 alendronate disodium (internal standard) were obtained from LGC Promochem India Pvt. Ltd (Mumbai, India) and Neucon Pharma Pvt. Ltd. (Goa, India) respectively. HPLC Grade acetonitrile and methanol were obtained from Merck, India. Deionized (18 MΩ /cm) water was generated in house using a Milli-Q System from Millipore (Bedford, MA, USA). All other solvents and reagents are of analytical grade and purchased from Merck, India or Spectrochem, India.

2.2. Preparation of Diazomethane

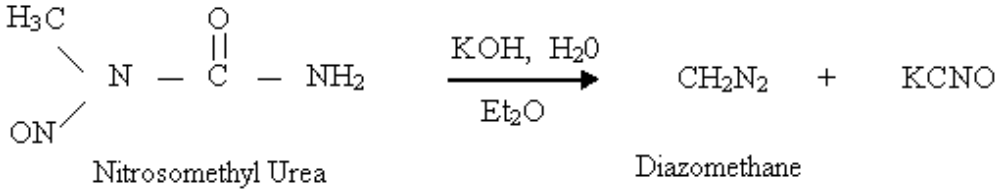
Diazomethane is a yellow gas at room temperature, liquefies at -23^{0}C, and freezes at -145^{0}C. It is extremely toxic and highly irritating gas which when inhaled in high concentrations can result in pulmonary edema. Long-term low- level exposure can lead to sensitization with asthma like symptoms. Furthermore, diazomethane is cited as carcinogens. Rough surfaces and strong light are known to detonate diazomethane. Keeping in view the toxicity of diazomethane, and as a preventive measure, all reactions pertaining its preparation and subsequent use are carried out in a chemical fume hood and behind a safety shield.

Methyl amine solution reacted with urea in presence of water and HCl to form methyl urea, which is nitrosated with sodium nitrite to give nitrosomethylurea (NMU).





Nitrosomethylurea (NMU) reacts with aqueous KOH in ether solution and forms diazomethane.



2.3. Preparation of standard stock solution of alendronate and internal standard

2.3.1. Preparation of alendronate stock solution

Alendronate working/reference standard equivalent to about 5 mg of alendronate is weighed and transferred into a 5 mL volumetric flask (plastic ware). Stock solution was prepared by dissolving the above content in water [Ultra pure/Type I or HPLC Grade] and made up the volume with the same to obtain a concentration of 1000000 ng / mL of alendronate. The above solution was diluted to about 50000 ng/mL with water [Ultra pure/Type I or HPLC Grade]. The stock solution was stored in refrigerator within 2-8°C.

2.3.2. Preparation of alendronate d6 disodium stock solution (ISTD – Internal Standard)

d6 alendronate disodium working/reference standard equivalent to about 5 mg of d6 alendronate disodium was weighed and transferred into a 5 mL volumetric flask (plastic ware). Stock solution was prepared by dissolving the above content in 2 % formic acid solution and made up the volume with the same to obtain a concentration of 1000 µg/mL of d6 alendronate. The stock solution was diluted with water [Ultra pure/Type I or HPLC Grade] to acquire about 50 µg/mL of intermediate ISTD dilution of alendronate d6 disodium. This intermediate ISTD dilution was also diluted with water [Ultra pure/Type I or HPLC Grade] to acquire about 1.0 µg / mL of alendronate d6 disodium. The stock, intermediate ISTD dilution and ISTD dilution were stored in refrigerator at 2-8°C.

2.4. Preparation of standards and quality control (QC) samples

Two separate primary stock solutions (with weights having a difference of less than 5% in LC-MS/MS) of alendronate and alendronate d6 disodium were stored in plastic vials and kept refrigerated (2–8 ^{0}C). The difference in the weighing of the two stock solutions is maintained so as to ensure

validity of the method. The stock solutions were then diluted with distilled water inorder to prepare various standard or quality control working solutions.

2.5. Sample preparation

Blank standard solution was prepared by adding 2% water to the screened blank human urine. The calibration standard solutions were freshly prepared by spiking 0.20 mL of the prepared standard working solutions into 10.0 ml of human urine.

Quality control sample solutions of low, medium and high levels were prepared by spiking the prepared quality control working solutions into urine. Finally 0.5 to 0.7 mL of each calibration curve and QC samples were aliquoted into different pre-labeled polypropylene-capped tubes and stored at -22 ± 5°C / –65 ± 10°C until analysis.

2.6. Extraction procedure

The required numbers of CC / QC samples were take out from the deep freezer and kept at room temperature for thawing. Before pipetting the samples were vortexed adequately to ensure complete mixing of contents. To a 500 µL aliquot of each CC / QC sample, 50 µL of ISTD working solution (about 1.0 µg / mL) and 50 µL of 5% ortho-phosphoric acid (v/v) were added and vortexed for one minute. After vortexing 500 µL of 10 mM potassium dihydrogen phosphate buffer was added and again vortexed for 1.0 minute.

The SPE extraction was carried out on [Orpheus] alumina basic 100 mg/1mL cartridges. Each cartridge was conditioned with 1.0 mL of methanol [HPLC grade] and 1.0 mL of 10 mM potassium dihydrogen phosphate buffer on SPE manifold applying low vacuum / pressure prior to sample loading. After the samples had been loaded onto the cartridges, 2 mL (1 mL X 2) of 10 mM potassium di-hydrogen phosphate was used for washing and the cartridges were dried under full vacuum/ pressure for 2 minutes. After that 0.5mL of freshly prepared diazomethane was added to each cartridge with respective prelabeled riavials under each cartridge. Diazomethane was allowed to elute form each cartridge into its respective prelabeled riavials. Cartridges were then eluted with 1.0 mL methanol [HPLC grade] into the same pre-labeled riavials. Extracts were dried under a gentle flow of nitrogen gas at 50°C temperature and reconstituted with 250 µL of mobile phase followed by vortexing of about 30 seconds.

2.7. Data Analysis

Analyst software Version 1.4.2 was used for the data acquisition and the evaluation of chromatographic data .The calibration plots of analyte peak area versus the analyte concentration were constructed by using the least square linear regression equation ($y = a + bx$).The criteria for acceptance for low, medium and high QC samples for inter-day and intra-day assay is a high correlation co-efficient (r^2) of >0.98, accuracy of ± 15 % of the nominal concentration and a precision of <15 % RSD

. The acceptance criteria for LLOQ sample is the same high correlation co-efficient and accuracy of ± 20 % of the nominal concentration and precision of <20 % RSD for the inter-day and intra-day assay.

The accuracy, sensitivity, precision, stability, recovery, reproducibility and reliability of the analytical method were confirmed by validation in accordance with the USFDA guidelines [99].

3. MATERIAL AND METHODS

3.1. Method Development

3.1.1. Mass Spectrometric Conditions

Ionization and fragmentation efficiency were the two main mass parameters which determines the detection or quantitation limit of alendronate compound. The ionization efficiency was typically a compound dependent parameter which was significantly influenced by the gas phase basicity or acidity in atmospheric pressure ionization. For electrospray, the important factor affecting the ionization efficiency was the mobile phase.

Methylation of alendronate by using diazomethane was employed inorder to enhance the sensitivity of the LC–MS/MS method for the quantification of alendronate. The mass spectrometry was operated in the positive ion electrospray mode. The temperature of heated capillary was set at 350 ^{0}C and its potential to 4.5 K.V. Nitrogen was used as a curtain gas and zero air was used as a turbo and nebulizer gas, set to 45 psi and 50 psi units respectively. The ultra pure nitrogen was used as a collision gas and pressure was set to 5 mtorr, subsequently collision energy was set to 32 eV for analyte and internal standard. Multi reaction monitoring (MRM) mode was employed and involved transition of the $[M-H]^+$ precursor ions to select ions at m/z 348 for drug and 354 for internal standard (IS) respectively. The half height mass peak was set to 0.7+ 0.1 amu (unit resolution) for both Q1 & Q3 and dwell time of 200 msec for each MRM channel.

In this method deuterated analyte was used as the internal standard. Stable isotope of alendronate i.e. d6 alendronate was used as internal standard to compensate for the potential matrix effects, caused by co-eluting endogenous components in biological fluids. The detrimental matrix effects have been identified as the primary cause for the failure of the quantitative bioanalytical LC-MS/MS method.

To optimize above mentioned parameters, alendronate and internal standard were tuned in development. A full scan electrospray positive ion mass spectrum was scanned and optimized. The mass and instrument dependent parameters were optimized at various conditions for drug and internal standard. A molecular ion was obtained by direct infusion of aqueous samples (500 ng/ml) at the flow rate of 10 μL/min. The Full scan spectra of alendronate showed $[M+Na]^+$, $[M+k]^+$ and $[M+NH_4]^+$ in addition to the $[M+H]^+$ ion, although the mobile phase contained no known sources of sodium, potassium and other ions. The overall relative response of the $[M+H]^+$ $[M+NH4]^+$ and $[M+Na]^+$ affected not only by presence of mobile phase additives (ammonium acetate) but also by the heated capillary temperature. These adduct ions were eliminated by changing the decluster potential from 25

V to 45 V and finally it was kept at 60 V for both and entrance potential from 9 V to 10 V. The condition was finally selected which favored the formation of the $[M+H]^+$ ions. It was essential to investigate the adduction of the ions because these ions were known to cause interference during spectral analysis. After investigation it was found that P-C-P structure and phosphonic group was responsible in producing adducts in the solution because they have a tendency to form chelate compounds. In this whole process glassware was not used because glassware was the main source of these adducts ions. The formation of dimmer and multimer in analyte spectra was also investigated (Table 1) and it was not observed in the scanned graph. It was paramount to evaluate the ruggedness of this mass spectral condition and consequently some more experiments were performed to evaluate the method performances which are being mentioned below:

Table 1. Optimization of mass spectrometry parameters and impact analysis

S.No	Experiment Name	Results
1	Dimmer	Not observed
2	Multimer	Not observed
3	Neutral Loss Scan	Not observed
4	Conjugation	Not observed
5	Sample stability	Stable at room temperature
6	Negative polarity	No significant m/z observed
7	Impurity identification	Not observed
8	Any degradation by product	No degradation products were observed

The fragmentation behavior of the $[M+H]^+$ ion was found to be unsatisfactory. Hence, ammonium acetate was used as the ionizing agent for monitoring the decay of the ammonium adduct ion. The method thus developed resulted in an assay with good sensitivity and produced linear calibration curves, but with poor repeatability and reproducibility. The primary cause for this phenomenon was the difference in the affinity of alendronate and the internal standard (d6 alendronate) for NH_4^+, Na^+ and K^+ and in the changing ratios of these adducts ions with time. An ionizing agent having a higher affinity for the analyte to the mobile phase thus resulted in producing reproducible calibration curves. Primary alkyl amine has a tendency to form hydrogen bonds with the oxygen atoms of the analyte and thus is known to suppress multimer formation and to reduce the Na^+ and K^+ effects.

MS/MS scan was performed to get product ion and fragmentation pattern of the molecule at different collision energy. Figure 1 represents the full scan and product ion mass spectra of alendronate. The corresponding exact mass of the fragment ion was found at m/z of 163.1. Same solution concentration 500 ng/ml directly was infused into the mass spectrometry at positive polarity eventually 163.1 was obtained as a sustainable ion. Fragmentation efficiency helps in determining the detection limit for a specific compound. Quantitative LC-MS/MS analysis was usually performed using the MRM mode, which helps in monitoring the decay of the protonated molecule to one specific fragment ion. Optimization of the collision energy (CE) was performed in order to specific fragment

ion. Collision induced dissociation of this ion results in an abundant fragment ion that were used for sensitive MRM analyses. Optimized values of compound related parameters and source gas parameters are summarized in Table 2.

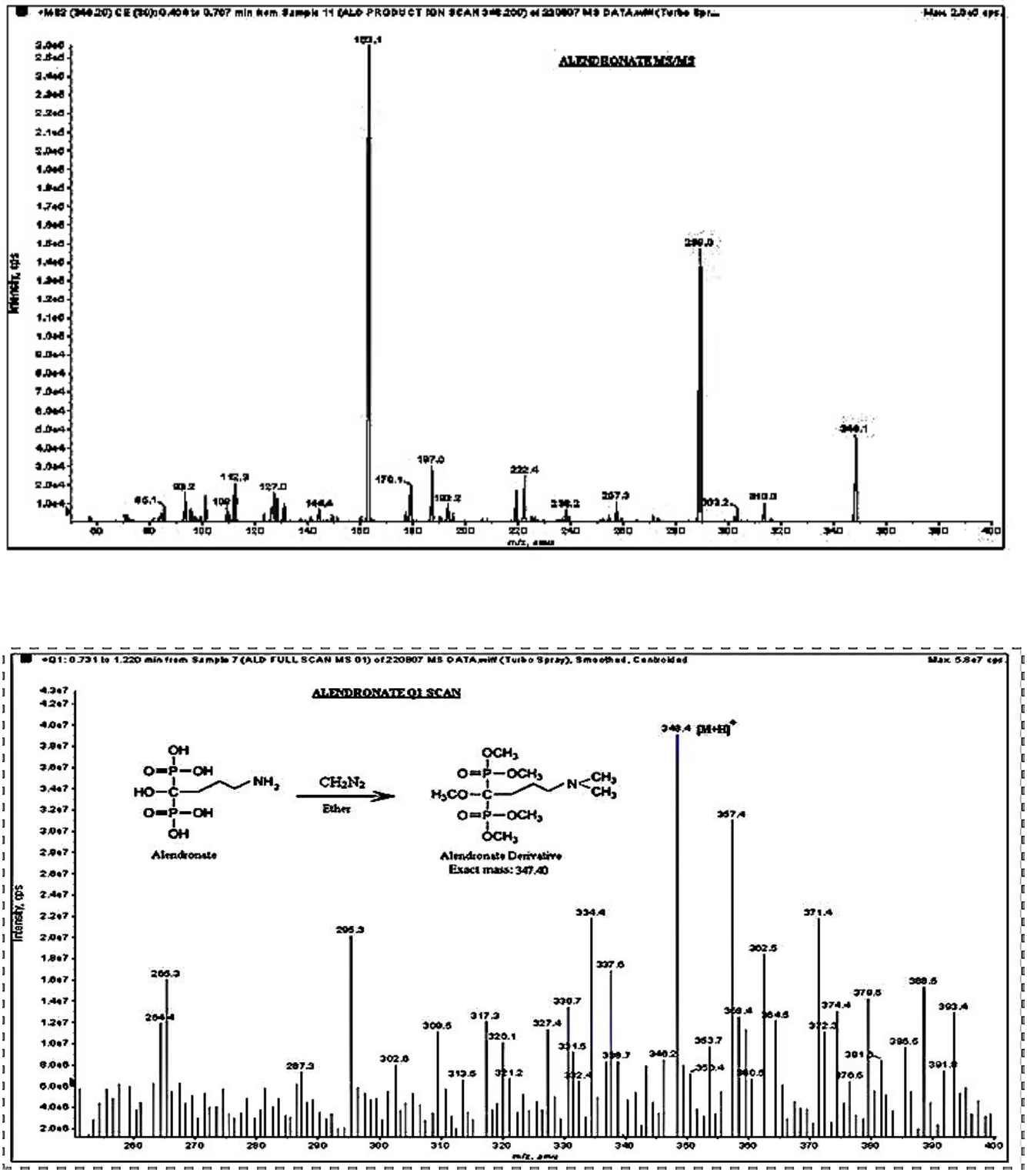


Figure 1. MS/MS scan and product ion mass spectra of alendronate

Table 2. Main working parameters for mass spectrometry

Parameter		Value
Curtain gas (psi)		20.00
Ion spray voltage (V)		2000.00
Temperature (^{0}C)		400.00
Nebulizer gas (psi)		60.00
Auxiliary gas (psi)		60.00
Collision activated dissociation (CAD gas)		6.00
Parameter	Alendronate	Alendronate d6 disodium (IS)
Declustering potential (V)	60.00	60.00
Entrance potential (V)	10.00	10.00
Collision Energy (V)	32.00	32.00
Cell exit potential (V)	13.39	10.00
Defluctering potential (V)	-25.0	-25.0
Channel electronic multiplier (V)	2200.0	2200.0
Ion transition m/z	348.00→163.1	354.00→168.00

3.1.2. Chromatographic Conditions

The separation of alendronate from the internal standard was accomplished by liquid chromatography (Shimadzu LC model 10ADVP integrated system). The system consists of an autosampler, a multichannel mobile phase degasser, a column heater and two pumps (Shimadzu, Columbia, MA, USA). Several different methods and modes of chromatography were optimized during method development including ion exchange, reversed phase, normal phase and ion pair chromatography. Alendronate is strongly polar and ionic and hence it was difficult to retain on the column and in addition to that alendronate has capacity to make complexation or chelation with metal ion and other cations of endogenous matrix which hampered the analysis. The selection of silica based anionic column and aqueous – organic mobile phase was selected to initiate the method development.

Subsequently after initial experiments it was decided to use reversed phase chromatography for method development, although alendronate and d6 alendronate (internal standard) are highly polar due to phosphonic group but after derivatisation it was converted from highly polar to moderately polar and this process increased hydrophobicity of the drug and internal standard and consequently the retention of analytes increased on the reversed phase column. Mobile phase used for separation of the analytes was acetonitrile: 40 mM ammonium acetate buffer (75:25, v/v). It was found that lower concentration of alendronate was sensitive enough when 40 mM concentration of buffer was used, even though high concentration of ammonium acetate also create problem during instrument operation for example precipitation on curtain plate. These conditions were further optimized for pH of buffer. Optimum buffering capacity occurred at pH equal to the pKa of the alendronate. The acidic mobile phase nature enhances the protonation of the drug and internal standard and simultaneously improved the sensitivity of the drug and internal standard. Alendronate is strongly ionic at moderate pH but after

derivatisation, pKa of compound got changed and hence ionic formation was also reequbilirate which was significantly helpful to retain compound on column. Chromatography of these anionic analyte was possible on SCX column and it was also maintained that irreversible reaction should not occur during actual sample analysis. The stationary phase (SCX) was modified into an ion exchange resin after often with quaternary ammonium functional group and these were suitable for the chromatography of relatively strong ionic species. The pH played a major role for retaining these ionic compounds at column so it was thoroughly planned to maintain ionic equilibration in the mobile phase system henceforth different pH from 2.0 to 6.5 were checked in mobile phase composition and critical points were insured against the sustainability of injection, reproducibility of the signal at interday and intraday, ruggedness, sensitivity, selectivity, specificity and retention of the drug and internal standard on column. The largest peak area for the alendronate was obtained when the on column solution pH was pH 4.0.

Biological samples particularly plasma and urine samples were likely to have pyrophosphate in the sample, which could possibly be an interference. Because a strong eluent condition of 40 mM ammonium acetate concentration was used, pyrophosphate elutes early and consequently did not interfere in the determination of alendronate. Furthermore initial reversed phase HPLC experiment showed that acceptable chromatography could be obtained for alendronate and internal standard in urine extracts using a Zorbax SCX column in addition to that flow rate was also optimized and it has significantly improved peak symmetry and chromatographic conditions. Earlier 0.6 mL/min of flow rate was taken but it was optimized and changed to 1.0 mL/min. Consequently in initial experiments, drug and internal standard peak width at half height was found to be 48 sec but after optimization of the flow rate, the peak area remarkably improved from 48 to 35 secs.

Alendronate intensity was significantly depending on the on column pH during derivatisation. Phosphonic ion is highly pH dependent. Column pH was checked and optimized. This is because alendronate sensitivity is totally dependent on its methylation during derivatisation. Derivatisation occurred at hepta level and hence it was only possible due to pH of adsorption phase when diazomethane was loaded to the column. Initially sample buffer pH was 7.8 but after optimization it was kept at 4.5. Furthermore the presence of alcohols affects the solubility of hydroxyl groups of alendronate. In some experiments it was noticed that the analyte methylation peak area was obtained with significant intensity when the pH of the solid phase column reaction was 3.5 at the loading time of diazomethane. Hence a washing buffer was used because it was a poor ligand for alendronate and it did not alter sensitivity significantly. Diazomethane concentration had a major impact on the ruggedness of the method. Diazomethane reaction could be performed efficiently on silica base anion cartridge sorbents. Underivatised alendronate binds strongly with quaternary ammonium ion and its interaction was mainly due to wander-walls, pi-pi bonding and hydrogen bonding. This bonding was disrupted by diazomethane when it was slowly added to the solid phase tube. It reacted with alendronate sites; subsequently methylation reaction started and it reacted on all available sites of the alendronate molecules. As a result it was converted from highly polar compound to moderately polar and hence it was easy to analyze alendronate at LC-MS/MS platform by employing reversed phase chromatography. However diazomethane volume was optimized to enhance the analyte signal. The volume was used from 250 μL to 1000 μL and it was found that there were very poor signal from 250

µL to 350 µL, but the signal was good at 400 µL to 1000 µL. Consequently most sensitive signal was observed at 500 µL which was also sustainable at different conditions. Attempt was made to keep diazomethane volume as much low as possible because diazomethane is highly toxic and explosive in nature; hence all reactions starting from its preparation to its use were carried out under a highly efficient chemical fume hood and behind a safety shield.

3.2. Method Validation

3.2.1. Precision and accuracy

Table 3. Precision and accuracy of quality control samples of alendronate

Alendronate	LOQ QC (ng/mL)	LQC (ng/mL)	MQC (ng/mL)	HQC (ng/mL)
Day 1				
n	8	8	8	8
Mean	2.0099	5.7208	285.4281	451.1509
±SD	0.03863	0.12042	3.53787	9.65508
Precision (% CV)	1.9	2.1	1.2	2.1
Accuracy (%)	99.4	94.8	95.2	94.0
Day 2				
n	8	8	8	8
Mean	1.9725	5.9290	284.9466	469.1934
±SD	0.05091	0.09756	1.88021	6.21901
Precision (% CV)	2.6	1.6	0.7	1.3
Accuracy (%)	97.6	98.2	95.0	97.8
Day 3				
n	8	8	8	8
Mean	1.8999	5.9150	287.4758	477.7411
±SD	0.04728	0.11642	3.76060	8.87260
Precision (% CV)	2.5	2.0	1.3	1.9
Accuracy (%)	94.0	98.0	95.9	99.6
Day 4				
n	8	8	8	8
Mean	2.0160	6.0531	289.598	479.6093
±SD	0.04036	0.08922	5.45301	11.29070
Precision (% CV)	2.0	1.5	1.9	2.4
Accuracy (%)	99.7	100.3	96.6	100.0
Day 5				
n	8	8	8	8
Mean	2.1641	6.0308	279.9893	464.1278
±SD	0.05521	0.08905	9.07409	16.94854
Precision (% CV)	2.6	1.5	3.2	3.7
Accuracy (%)	107.0	99.9	93.4	96.7
Global Statistics				
Mean	468.3645	285.4876	5.9297	2.0125
±SD	14.86000	6.00616	0.15432	0.09810
Precision (% CV)	3.2	2.1	2.6	4.9
Nominal value (ng / mL)	479.838	299.899	6.036	2.022
Accuracy (%)	97.6	95.2	98.2	99.5
n	40	40	40	40
Acceptance criteria: Precision (%CV) should be $\leq$ 15% (For LOQ QC <20 %) Accuracy (%Nominal) should be within 85-115% (For LOQ QC 80-120%)				

-Precision

Precision of the assay was measured by determining the coefficient of variance (%) over the concentration range of limit of quantification, low, medium and high quality control samples of alendronate. The Intra-day (within-batch) ranged from 0.7 % to 3.7 % whereas, the inter-day (between-batch) ranged from 2.1 % to 4.9 %.

-Accuracy

Accuracy of the assay was defined as the absolute value of the ratio of the mean back-calculated values of the quality control samples to their respective nominal values, expressed in percentage. The Intra-day accuracy (within-batch) ranged from 93.4 % to 107.0 % as evident from Table 3 and the Inter-day accuracy (between-batch) ranged from 95.2 % to 99.5 %.

3.3. Total recovery

The total recovery was determined by comparing the detector response obtained from extracted alendronate samples at three levels of low, medium and high QC samples with that obtained from the un-extracted aqueous QC samples. The mean recovery for the low, medium and high quality control samples were found to be 97.6 %, 97.0 % and 98.7 % respectively.

3.4. Robustness

The unaffected small changes in method validation parameters (i.e. different column of same make and processed by different analyst) were carried out deliberately during method validation. These parameters were verified simultaneously as a part of factorial experiment. The robustness was established by performing precision and accuracy batch processed by different analyst and performed using different column of same make. The within batch precision of quality control samples was 2.0 % to 5.4 % which was within the acceptance criteria of ≤ 15 % for all quality control samples except for LOQ QC which was ≤ 20 %. The within batch accuracy of quality control samples was 94.1 % to 98.5 %, which was within the acceptance criteria of 85 – 115 % of nominal for all quality control samples except for LOQ QC which was 80-120 %.

3.5. Matrix effect

Matrix effect was evaluated by preparing low and high quality control samples in six different blank urine batches and analyzed using a freshly processed calibration curve standards prepared for this validation. Precision of low and high quality control samples were 13.8 % and 6.7 % respectively, which was within the acceptance criteria of ≤15 %. Accuracy of low and high quality control samples were 91.9 % and 97.0 % respectively, which was within acceptance criteria of 85-115 % of nominal. In the present study, the determination of alendronate is not affected by co-extracted matrix components under the LC–MS/MS conditions used.

3.6. Linearity and goodness-of-fit

A linear equation was judged to produce the best fit for the concentration vs. area response relationship.

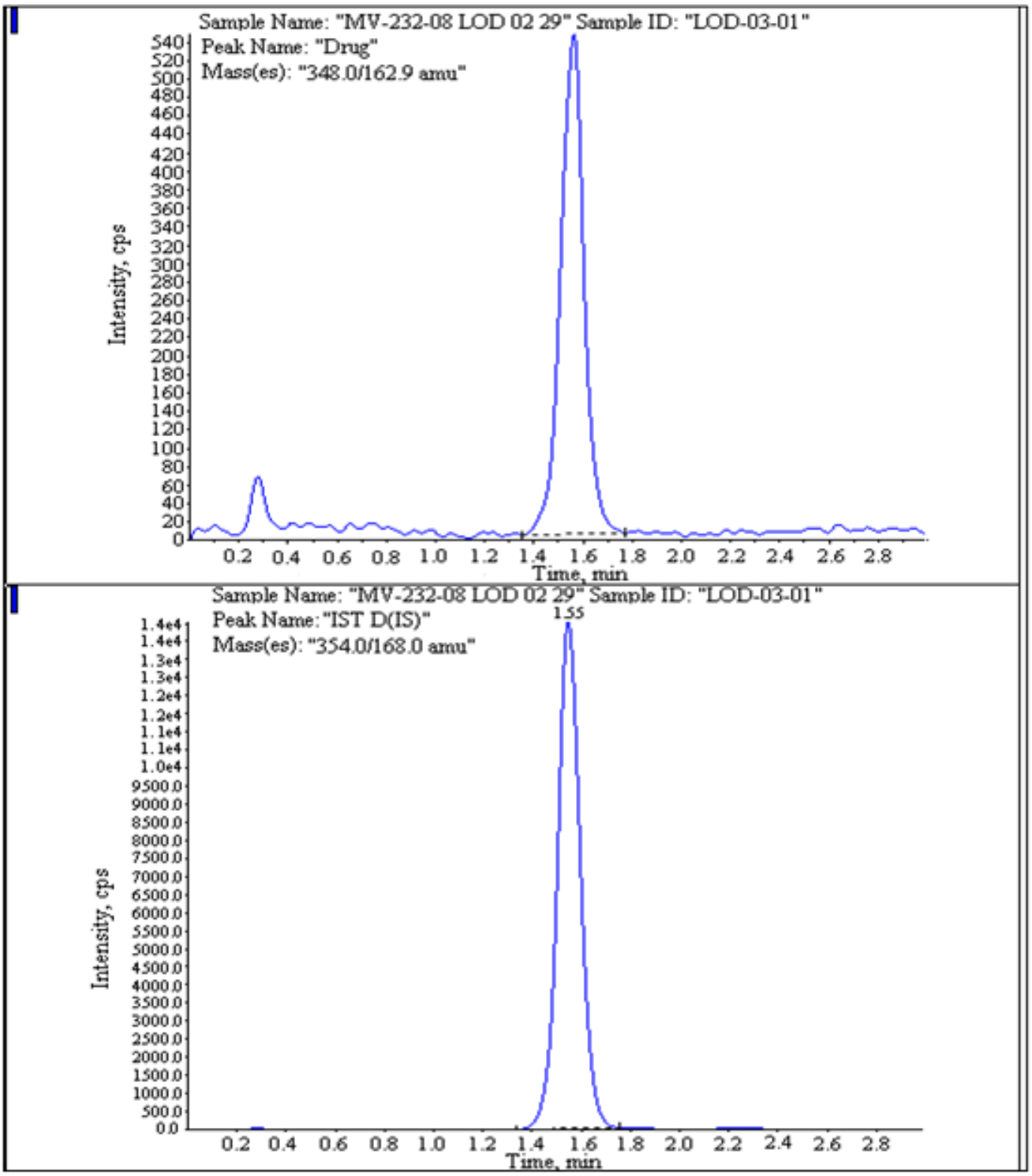


Figure 2. The limit of detection of the method

The regression type was 1/concentration² and peak area ratio for an 8-point calibration curve was found to be linear from 2.000 ng/mL to 600.381 ng/mL. The goodness of fit (r^2) was consistently greater than 0.99 during the course of validation. Calibration curve was processed and run along with each batch that was analysed on consecutive days using freshly prepared solution each day. The between-batch variability in the calibration curves was reported with the coefficients of variation (CVs) and accuracy obtained during method validation. The range of precision and accuracy of the back-calculated concentrations of the standard curve points was from 1.2 % to 2.3 % and from 97.1 % to 105.5 % respectively.

3.7. Limit of detection

Detection limit was established by means of analysis of samples of known and decreasing concentrations of drug up to the lowest detectable level. The limit of detection of the method was 0.250 ng/mL (Figure 2).

3.8. Re-injection reproducibility

Re-injection reproducibility for alendronate was determined by re-injecting eight sets of two distinct levels of low and high quality control samples of P&A I batch. The percent mean ratio of the back calculated values for the low and high quality control samples was 101.1 % and 99.4 % respectively, which was within the acceptance range of 90–110 % while comparing the mean of the back-calculated values against the mean of values obtained from an original injection.

3.9. Stability of urine sample during storage

The freeze and thaw stability of alendronate for low and high quality control samples was determined by storing them in the freezer maintained at – 65 ± 10°C and analyzing after three freeze thaw cycles using a set of freshly prepared calibration curve standards. The percent degradation was determined by comparing the mean of the back-calculated values from the stability samples with the nominal values and with the mean of freshly processed samples. The percent change of low and high quality control samples was -0.9 % and – 4.4 % respectively after three cycles, which was within the acceptance criteria of ±15 %. The percent mean ratio of the low and high quality control samples was 98.9 % and 98.4 % respectively, which was within the acceptance range of 90–110 %, while comparing the result with freshly processed quality control samples. Bench top stability of alendronate for low and high quality control samples was evaluated by analyzing them using a set of freshly prepared calibration curve standards. The stability samples were kept on bench at room temperature for 7.0 hours. The percent degradation was determined by comparing the mean of back-calculated values of the stability samples with the nominal values and with the mean of freshly processed samples. The percent change of low and high quality control sample was –2.2 % and –2.3 % respectively, which was within the acceptance criteria of ±15 %. The percent mean ratio of the low and high quality control

samples was 97.6 % and 100.5 % respectively (Table 4), which was within the acceptance range of 90–110 %, while comparing the result with freshly processed quality control samples.

Table 4. Freeze/thaw, Bench top stability and re-injection reproducibility of alendronate

Freeze and thaw stability				
	Freshly Processed		After three cycles	
	HQC	LQC	HQC	LQC
	Back-calculated value (ng / mL)			
Mean	466.143	6.0501	458.748	5.9816
Nominal value (ng /mL)	479.838	6.036	479.838	6.036
N	8	8	8	8
% Change			-4.4	-0.9
% Mean ratio			98.4	98.9
Bench top stability				
	Freshly Processed		After 7.0 hours	
	HQC	LQC	HQC	LQC
	Back-calculated value (ng / mL)			
Mean	466.143	6.0501	468.684	5.9029
Nominal value (ng /mL)	479.838	6.036	479.838	6.036
N	8	8	8	8
% Change			-2.3	-2.2
% Mean ratio			100.5	97.6
Bench top stability				
	Original run		Re-injected run	
	HQC	LQC	HQC	LQC
	Back-calculated value (ng / mL)			
Mean	451.151	5.7208	448.27	5.7853
Nominal value (ng / mL)	479.838	6.036	479.838	6.036
N	8	8	8	8
% Mean ratio			99.4	101.1

4. APPLICATION OF THE METHOD

The validated method has been successfully used to analyze samples obtained after the administration of a single dose of 75 mg alendronate tablets to healthy volunteers participating in bioavailability studies.

The urine concentration of all the volunteers at times 1.5-1 hrs as predose and 0-15min, 15min-1h, 1-2h, 2-3h, 3-4h, 4-6h, 6-8h, 8-12h, 12-24h, 24-36h and 36-48h post dose in each of two periods of test and reference were determined after the analysis using HPLC-MS/MS.

Table 5. Mean urine concentration of reference and test formulation

Pk Parameter	Unit	N	Reference Formulation		Test Formulation	
			Mean	SD	Mean	SD
Cmax	ng/mL	75	675.376	258.308	670.843	254.0848
AUC0-t	ng*hr/mL	75	4575.885	963.9147	4497.887	1208.5517
AUC0-inf	ng*hr/mL	75	5738.452	1397.588	5666.536	1583.818

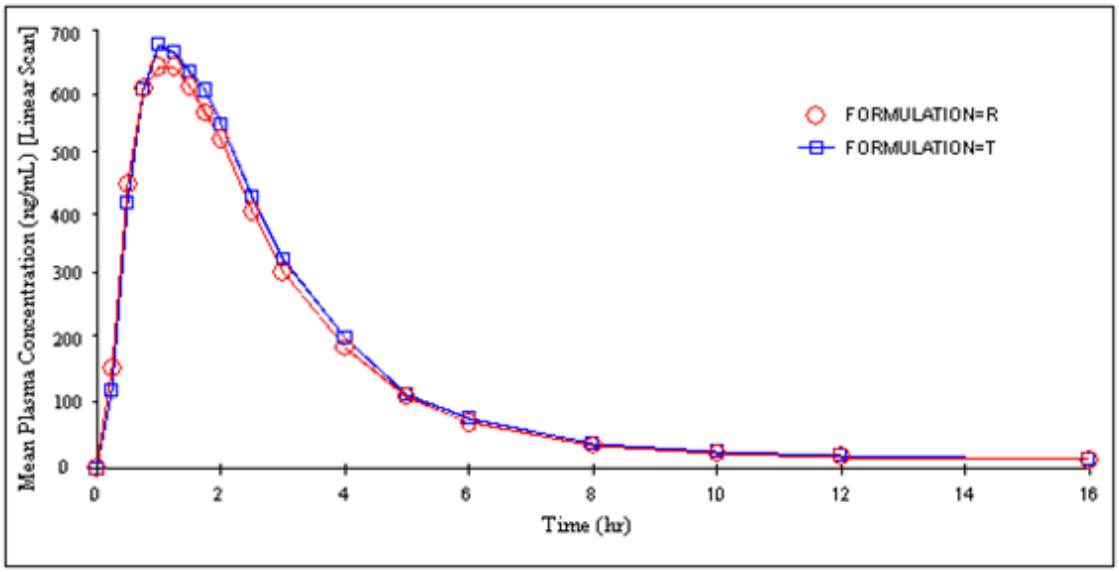


Figure 3. The mean urine concentration versus time profile

The maximum mean concentration of the test and reference products were 675.4 ± 258.30 and 670.8 ± 254.08 ng/mL, occurring at 1.55±0.35 h and 1.61±0.04 h. Pharmacokinetic parameters were calculated from the subjects who have successfully completed period I and II. Presented concentrations were converted in to R_{max} calculation based on urine discharge. The mean urine concentration versus time profile (Table 5) is shown in Fig. 3.

5. CONCLUSIONS

For the first time, a highly sensitive and reliable bioanalytical method for the determination of alendronate in human urine was developed and validated using on cartridge derivatisation with liquid chromatography tandem mass spectrometric detection. The derivatisation improves the separation and ionization efficiency. Moreover, the generated derivatives give particular product ions by CID (collision induced dissociation). The use of chemical derivatisation of alendronate with diazomethane not only made the analysis possible but also after some optimization, it enhanced the detection sensitivity of the analyte in electrospray ionization. Derivatisation also offers the option of altering the

major mass spectral peaks to avoid interference. When derivatisation is done on column, it can provide an alternative confirmation or identical method for particular functional groups.

The method has shown acceptable precision, accuracy and adequate sensitivity for use in bioequivalence studies of alendronate in healthy volunteers. The method described here is simple, sensitive, specific and fully validated as per FDA guidelines [76]. The validated method allows quantification of alendronate in concentration range of 2-600 ng/ml. Thus, liquid chromatography tandem mass spectrometry with on cartridge derivatisation of analyte can provide a very useful technique for the analysis of less sensitive analytes.

References

1. W. Jeal, L.B. Barradel, D. McTavish, *Drugs* 53(1997)415.
2. M.Sato, W. Grasser, N.Endo, *J. Clin. Invest*. 88(1991)2095.
3. J.E. Fisher, M.J. Rogers, J.M. Halasy, *Proc. Nat. Acad. Sci*. 96(1999)133.
4. S.P. Luckman, D.E. Hughes, F.P. Coxon, *J. Bone Miner. Res*. 13(1998)581.
5. E. Beek, C. Lowik, G. Der, *J. Bone Miner. Res.* 14(1999)722.
6. G.A. Rodan, H.A. Fleisch, *J. Clin. Invest.* 97(1996) 2692.
7. O. Fromigue, J.J. Body, *J. Endocrinol. Invest*. 25(2002) 539
8. G.I. Im, S.A. Qureshi, J. Kenny, H.E Rubash, A.S. Shanbhag. *Biomaterials* 25(2004)4105.
9. G.G. Reinholz, B. Getz, L. Pederson, *Cancer Res.* 60(2000) 6001.
10. L.I. PlotkinI, R.S. Weinstein, A.M. Parfitt, *J. Clin. Invest*. 104(1999)1363.
11. J.D. De Marco, S.E. Biffar, D.G. Reed, M.A. Brooks, *J. Pharm. Biomed. Anal.* 7(1989)1719
12. W. Kline, *J. Chromatogr*. 534(1990) 139.
13. W. Kline, B.K. Matuszewski, *J. Chromatogr*. 583(1992) 183.
14. P. Ptacek, J. Klima, J. Macek, *J. Chromatogr. B* 767(2002)111.
15. M.J. Lovdahl, D.J. Pietrzyk, *J. Chromatogr. A* 850(1999)143.
16. E. Kwong, A.M. Chiu, S.A. McClintock, M.L. Cotton, *J. Chromatogr. Sci.* 28(1990) 563.
17. R. Yieng, Z. Xue, *J. Chromatogr. A* 719(1996) 345.
18. E.W. Tsai, M.A. Brooks, *J. Chromatogr.* 596(1992)217.
19. E.W. Tsai, S.D. Chamberlin, R.J. Forsyth, C. Bell, D.P. Ip, M.A. Brooks, *J. Pharm. Biomed. Anal*. 12(1994)983.
20. X.Z. Qin, E.W. Tsai, T. Sakuma, D.P. Ip, *J. Chromatogr. A* 686(1994)205).
21. P. Ptacek, J. Klima, J. Macek, *J. Chromatogr. B.* 767(2002)111.
22. D.G. Reed, G.P. Martin, J.M. Konieczny, M.A. Brooks, *J. Pharm. Biomed. Anal.* 13(1995)1055.
23. O. Abdel Razak, S.F. Belal, M.M. Bedair, R.S. Haggag , *Talanta* 59(2003)1061.
24. S.K. Aldeeb, II HamdanI, S.M. Al Najjar, *Talanta* 64(2004) 695.
25. A. K. Jain, V. K. Gupta and L. P. Singh, Analytical *Proceedings including Analytical Communications*, 32 (1995) 263.
26. A. K. Jain, V. K. Gupta, B. B. Sahoo and L. P. Singh, *Analytical Proceedings including Analytical Communications*, 32 (1995)99.
27. S. K. Srivastava, V. K. Gupta and S. Jain, *Analyst*, 120 (1995) 495.
28. S. K. Srivastava, V. K. Gupta, M. K. Dwivedi and S. Jain, Caesium, *Analytical Proceedings including Analytical Communications*, 32 (1995) 21.
29. A. K. Jain, V. K. Gupta and L. P. Singh, *Bull. Electrochem.* 12(1996)418
30. S. K. Srivastava, V. K. Gupta and S. Jain, *Electroanalysis*, 8 (1996) 938

31. S. K. Srivastava, V. K. Gupta and S. Jain, *Anal. Chem*. 68(1996)1272.
32. A. K. Jain, V. K. Gupta, L. P. Singh and U. Khurana, *Electroanalysis*, 9(1997)1360.
33. V. K. Gupta, A. K. Jain, L. P. Singh and U. Khurana, *Anal. Chim. Acta*, 355(1997)33.
34. A. K. Jain, V. K.Gupta, U. Khurana and L. P. Singh, *Electroanalysis*, 9 (1997) 857.
35. A. K. Jain, V. K. Gupta, L. P. Singh and U. Khurana, *Analyst*, 122(1997) 583.
36. V. K. Gupta, S. Jain and U. Khurana, *Electroanalysis*, 9 (1997) 478
37. V. K. Gupta and P. Kumar, *Anal. Chim. Acta*, 389(1999) 205
38. V. K. Gupta, A. K. Jain, L. P. Singh and U. Khurana, *Sens. Actuators B*, 55(1999)201
39. V.K. Gupta, R. Mangla, U. Khurana and P. Kumar, *Electroanalysis*, 11 (1999)573
40. V. K. Gupta, R. Prasad, R. Mangla, and P. Kumar, *Anal. Chim. Acta*, 420(2000)19
41. V.K.Gupta, Rajni Mangla and S. Agarwal, *Electroanalysis*, 14, (2002) 1127
42. V. K. Gupta, R. Prasad, A. Kumar, *J. Appl. Electrochem.*, 33(2003)381.
43. V. K. Gupta, R. Prasad, A. Kumar, *Talanta*, 60(2003)149.
44. V.K Gupta, S. Jain, S. Chandra, *Anal. Chim. Acta* , 486(2) (2003)199.
45. V. K. Gupta, R. Prasad and Azad Kumar, *Talanta*, 63(2004)1027
46. R. Prasad, V. K. Gupta and Azad Kumar, *Anal. Chim. Acta* , 508(2004)61
47. R.N. Goyal, V.K. Gupta, M. Oyama and N. Bachheti, *Electrochem. Commun.*, 7 (2005)803
48. R.N. Goyal, V.K. Gupta, A. Sangal and N. Bachheti, *Electroanalysis*, 17 (2005)2217.
49. V. K. Gupta, S. Chandra and H. Lang, *Talanta*, 66(2005)575
50. V. K. Gupta, R. Ludwig and S. Agarwal, *Anal. Chim. Acta*, 538 (2005)213
51. A. K. Jain, V. K. Gupta, L. P. Singh, P. Srivastava and J. R. Raisoni, *Talanta*, 6(2005)716
52. A.K. Jain, V.K. Gupta, S. Radi, L.P. Singh, J.R. Raisoni, *Electrochim. Acta*,51(2006) 2547
53. V. K. Gupta, A. K. Singh and Barkha Gupta, *Anal. Chim. Acta*, 575 (2006)198
54. R.N. Goyal, V.K. Gupta, A. Sangal and N. Bachheti, *Electrochem. Commun.*,8 (2006)65
55. A.K. Singh, V. K. Gupta and Barkha Gupta, *Anal. Chim. Acta*, 585(2007)171
56. V. K. Gupta, A. K. Jain and G. Maheshwari, *Int. J. Electrochem. Sci.*, 2 (2007) 102.
57. V. K. Gupta, A. K. Singh, M. Al Khayat, B. Gupta, *Anal. Chim. Acta*, 590(2007)81
58. V.K. Gupta, A.K. Jain, M. Al Khayat, S. K. Bhargava, J.R. Raisoni, *Electrochim. Acta*, 53(2008)5409
59. R.N. Goyal, V.K. Gupta and S. Chatterjee, *Electrochim. Acta*, 53 (2008)5354
60. R.N. Goyal, V.K. Gupta, N. Bachheti and R.A. Sharma, *Electroanalysis*, 20 (2008)757.
61. V. K. Gupta, R. N. Goyal, and R. A. Sharma, *Int. J. Electrochem. Sci.* 4 (2009) 156.
62. V. K. Gupta, M. Al Khayat, A.K. Singh and Manoj. K. Pal, *Anal.Chim. Acta*, 634(2009)36
63. R.N. Goyal, V.K. Gupta and S. Chatterjee, *Biosens. Bioelectron.*, 24 (2009)1649
64. Vinod K. Gupta, Rajeev Jain, Manoj K. Pal, *Int. J. Electrochem. Sci.*, 5 (2010) 996
65. V. K. Gupta, A. J. Hamdan, R. Jain, S. Agarwal, A. K. Bharti, *Anal. Chim. Acta*, 681(2010) 27
66. R. Jain, V.K. Gupta, N. Jadon and K. Radhapyari, *Anal. Biochem.*, 407 (2010)79.
67. V.K. Gupta, *The Arabian Journal for Science and Engineering A-Science 35(2A*) (2010) 7
68. R.N. Goyal, V.K. Gupta and S. Chatterjee, *Sens. Actuators B*, 149 (2010)252
69. V. K. Gupta, R. Jain, K. Radhapyari, N. Jadon, Shilpi Agarwal, *Anal. Biochem*,408(2011)179
70. V.K. Gupta, S. Agarwal, B. Singhal, *International Journal of Electrochemical Science*,6(2011) 3036-3056
71. V.K. Gupta, A. Nayak, S. Agarwal, B. Singhal, *Combinatorial Chemistry and High Throughput Screening*,14(2011)284-302
72. V.K. Gupta, R. Jain, M.M. Antonijevic, H. Khani, M.N. Siddiqui, A. Dwivedi, R. Mishra, S. Agarwal, *International Journal of Electrochemical Science*,6(2011)37-51
73. V.K. Gupta, I. Ali, *Separation and Purification Technology* 18 (2000)131-140
74. I. Ali, V.K. Gupta, *Nature Protocols* 1 (2007) 2661-2667
75. V.K. Gupta, P.J.M. Carrott, M.M.L. Ribeiro Carrott, Suhas, *Critical Reviews in Environmental Science and Technology* 39(2009) 783-842

76. V.K.Gupta, D. Mohan, S. Sharma, *Separation Science and Technology* 33(1998)1331-1343
77. A. Mittal, L. Kurup, V.K.Gupta, *Journal of Hazardous Materials*,117(2005)171-178
78. V.K.Gupta, I. Ali, *Environmental Science and Technology*,42(2008)766-770
79. V.K.Gupta, A. Rastogi, *Colloids and Surfaces B: Biointerfaces*, 64 (2008)170-178
80. V.K.Gupta, A.Rastogi, *Journal of Hazardous Materials*, 154 (2008)347-354
81. V.K.Gupta, A. Rastogi, M.K. Dwivedi, D. Mohan, *Separation Science and Technology*, 32 (1997), 2883-2912
82. V.K. Gupta, A. Mittal, V. Gajbe, J. Mittal, *Industrial and Engineering Chemistry Research*, 45 (2006)1446-1453
83. V.K.Gupta, I. Ali, V.K. Saini, *Journal of Colloid and Interface Science*,315 (2007)87-93
84. V.K. Gupta, R. Jain, S. Varshney, *Journal of Hazardous Materials*,142(2007)443-448
85. V.K. Gupta, A. Rastogi, *Journal of Hazardous Materials*,153(2008)759-766
86. V.K. Gupta, R. Jain, A. Mittal, M. Mathur, S. Sikarwar, *Journal of Colloid and Interface Science*, 309 (2007)464-469
87. V.K. Gupta, A. Rastogi, *Journal of Hazardous Materials*,163(2009)396-402
88. V.K. Gupta, A. Mittal, L. Kurup, J. Mittal, *Journal of Colloid and Interface Science*, 304 (2006)52-57
89. V.K. Gupta, R. Jain, S. Varshney, *Journal of Colloid and Interface Science*, 312 (2007)292-296
90. V.K.Gupta, I. Ali, V.K. Saini, *Water Research*, 41(2007)3307-3316
91. V.K. Gupta, R.N. Goyal, R.A. Sharma, *Analytica Chimica Acta*, 647 (2009) 66-71
92. V.K. Gupta, A. Rastogi, A. Nayak, *Journal of Colloid and Interface Science*,342(2010)135-141
93. V. K. Gupta, I. Ali and Shilpi Agarwal, *Int. J. Electrochem. Sci.*, 6(2011) 5639-5648.
94. V. K. Gupta, M. R. Ganjali, Hadi Khani, P. Norouzi, A. Nayak, Shilpi Agarwal, , Critical Reviews in Analytical Chemistry, 41, (4) (2011)1-32.
95. B.J. Gertz, P. Shao, D.A. Hanson, H. Quan, S.T. Harris, H.K. Genant, C.H. Chesnut, D.R. Eyre, *J. Bone Mineral Res.* 9(1994)135.
96. R. Endele, H. Loew, F. Bauss, *J. Pharm. Biomed. Anal.* 39(2005)246.
97. P.T. Vallano, S.B. Shugarts, W.F. Kline, E. Woolf, B.K. Matuszewski, *J. Chromatogr*. 794(2003)23.
98. I. Tarcomnicu, M.C. Gheorghe, L. Silvestro, S.R. Savu, I. Boaru, A. Tudoroniu, *J. Chromatogr. B.* 877(2009)3159.
99. Guidance for industry, Bioanalytical Method Validation. US Department of Health and Human Services. Food and Drug Administration. Centre for Drug Evaluation and Research, Rockville, MD, 2001.