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Determination of Alendronate in Human Plasma By using Ultra-Performance Liquid Chromatography-Mass Spectrometry/ Mass Spectrometry.

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ABSTRACT
INTRODUCTION
The process of determining if a quantitative analytical method is adequate for biochemical applications is known as bioanalytical method validation (BMV).Alendronate is a medication that is used to prevent and treat osteoporosis in humans. Osteoporosis causes bones to shrink and fracture more easily. Osteoporosis is more likely to develop as you become older, after menopause, or if you use corticosteroid drugs (such as prednisone) for a long time. The aim of our work is to develop a validated method for the determination of Alendronate in plasma by using LC-MS/MS.
MATERIALS AND METHODS
The alendronic acid D6 is an internal solution (IS). Separation was achieved by liquid-liquid extraction on Luna 3a HILIC 200A (100 mm×2.0 mm), 2.5 µm column. The mixture of Acetonitrile: Buffer-1 in the ratio of 70:30%, v/v is used as a mobile phase 0.4 ml/min is used as a flow rate.
RESULT
The calibration curve is linear and plotted. The developed method is an accurate, precise, sensitive method for the determination of alendronate from human plasma solution. The reported method is suitable for bioequivalence and pharmacokinetic studies.

Keywords: Bio-analytical method,Alendronate, Osteoporosis, Human plasma, UPLC-MS/MS, Validation.

I. INTRODUCTION

A bioanalytical method is a series of procedures for collecting, processing, storing, and evaluating a biological matrix with the purpose of determining the presence of a chemical molecule. The process of determining if a quantitative analytical method is adequate for biochemical applications is known as bioanalytical method validation (BMV).[1-2]All processes that demonstrate that a particular method developed and utilized for quantitative measurement of analytes in a given biological matrix is reliable and reproducible are included in bioanalytical method validation.[3]The process of validating a bioanalytical technique includes determining whether the method's performance characteristics fulfill the requirements for the intended bioanalytical application. These performance characteristics are stated in terms of validation parameters for bioanalytical methods.[4-5] Precision and accuracy, as well as sensitivity, are important validation characteristics for bioanalytical methods.Its scope is to demonstrate the validity of the method Validation of bioanalytical methods carried out during the development and implementation of a novel bioanalytical method.[6-8]

The term UPLC, meaning "Ultra Performance Liquid Chromatography," was introduced by Waters Corporation when they introduced their Acquity LC system.The UPLC is based on the principle of use of a stationary phase consisting of particles less than 2 (µmwhile HPLC columns are typically filled with particles of 3 to 5 µm).Small particles gave increased speed, improved resolution, and more sensitivity to the fundamental instrument in the UPLC process. To fulfill the aim, a design with advanced technology in the pump, autosamplers, detector, data system, and service diagnostics as needed.[9-10]

Alendronate is a medication that is used to prevent and treat osteoporosis in humans. Osteoporosis causes bones to shrink and fracture more easily.Although risedronate is the recommended agent in males with osteoporosis,

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alendronate is a viable choice for preserving or growing bone mass.[11-12]Bisphosphonates include alendronate. Binding to hydroxyapatite crystals in bone slows osteoclast-mediated bone resorption and reduces bone matrix breakdown.[13]Three randomised, placebo-controlled studies investigated the antifracture effectiveness and safety of daily given alendronate for the treatment of postmenopausal osteoporosis.[14-15]

II. MATERIALS AND METHODS

The raw materials of Alendronate and alendronate D6 are procured from MTR lab, Chennai. The samples are authenticated by melting and solubility studies. UPLC method has given liquid chromatography a new lease of life. It may be used on particles with a diameter of less than 2mm to improve resolution, speed, and sensitivity. The human plasma was collected from human volunteers. The methanol and water used in HPLC grade. This method helps to reduce the cost and time consumption. The structure and profile details refer to Table 1 and Figure 1.

Chromatographic conditions:
UPLC Conditions:Column used Luna3u, HILIC 200A,(100 mm x 2.00 mm)
Mobile Phase Acetonitrile: Buffer-1 (70:30%,v/v)
Injection volume:10 uL
Flow rate:0.4 ml/min
Column oven temperature: 40 C
Retention Time: Alendronate :1.27±0.3min, Alendronate Acid D6: 1.27±0.3min
Total run time:2.5 min.
Mode of ionization: ES +

Mass chromatography parameter

Acquire chromatograms using the computer Mass lynx V4.l SCN 876software version supplied by Waters Inc and the desolvation temperature is 450°C. The desolvation gas flow is 1000L/hr, Capillary voltage is 3.50 kV and the cone gas flow: 150 L/hr. Refer Table 2.

Preparation of Reagent
Buffer-1: 110 mM Ammonium Acetate Solution]

Weigh about 770.80 mg of Ammonium Acetate in 1000 mL of reagent bottle containing 500 mL of water and make up the volume to 1000 mL with purified water. Mix well and sonicate in ultrasonic bath for few minutes. Provide a batch number and complete 'Solution Preparation Form'. Use this solution for four days from the date of preparation.

Buffer-2: [10 mM Potassium Di-hydrogen Phosphate]:

Weigh about 1.36 gm of Potassium Di-hydrogen Phosphate in 1000 mL of reagent bottle containing 500 mL of water and make up the volume to 1000 mL with purified water, Mix well and sonicate in ultrasonic bath for few minutes. Provide a batch number and complete Solution Preparation Form'. Use this solution for four days from the date of preparation.

Mobile Phase: [Acetonitrile: Buffer-I (10:30, % v/v)

Measure 700 mL of Acetonitrile and transfer into 1000 mL reagent bottle and add 300 mL of buffer-I. Mix well and sonicate in ultrasonic bath for few minutes. Provide a batch number and complete 'Solution Preparation Form [s]. Use this solution for four days from the date of preparation.

Diluent: [Acetone-M: Water (50:50, % v/v)]

Measure 500 mL of Methanol and transfer into 1000 mL reagent bottle and add 500 mL of Water.Mix well and sonicate in ultrasonic bath for few minutes. Provide a batch number and complete 'Solution Preparation Form'. Use this solution tör four days from the date of preparation.

Strong Wash Solution: [Acetonitrile: Water (80:20, %v/v)]

Transfer 800 mL of Acetonitrile into 1000 mL reagent bottle containing 200 mL of water. Mix well and sonicate in ultrasonic bath for few minutes. Provide a batch number and complete 'Solution Preparation Form'. Use this solution for four days from the date of preparation.

Weak Wash Solution: [Acetonitrile: Water (50:50, % v/v)]

Transfer 500 mL of Acetonitrile into 1000 mL reagent bottle containing 500 mL of water. Mix well and sonicate in ultrasonic bath for few minutes. Provide a batch number and complete 'Solution Preparation Form'. Use this solution for four days from the date of preparation.

Preparation of Analytical Solutions
Alendronate Stock Solution for CC (1 mg/mL)

Weigh accurately about 2 mg of equivalent to Alendronate transfer into 2 mL volumetric flask and add ImL water to dissolve and make up to the volume with water, Calculate the

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final concentration of Alendronate in pg/mL as follows:

$$\frac{\text{weight of Alendronate in mg}}{2\text{ml}} \times \frac{\text{potency}}{100} \times \frac{M1}{M2} \times 1000$$

Where. Ml is the molecular weight of Alendronate and M2 is the molecular weight of Alendronate salt.

Alendronate Stock Dilution :

Prior to spiking, prepare stock dilutions of Alendronate by using diluent in the concentration range as mentioned in the Table 3 below:-

Spiked Calibration Curves for Standards

Transfer 0.2 mL of the stock aliquot of corresponding concentrations of the above mentioned stock dilutions of Alendronate into 10 mL volumetric flask and make up the volume with K2EDTA plasma to achieve the following calibration curve concentrations mentioned in the **Table 4.**Pipette 0.600 mL aliquot of each calibration spiked standard into polypropylene-capped tubes and store it under 70°C±1 S °C.

Preparation of Internal Standard Stock Solution:

Weigh accurately about 2 mg of Alendronic acid D6 transfer into 2 mL volumetric flask and add I mL water to dissolve and make up to the volume with water. Calculate the final concentration of Alendronic acid D6 in gg/mL as follows:

$$\frac{\text{weight of Alendronic acid D6 in mg}}{2\text{ml}} \times \frac{\text{potency}}{100} \times \frac{M1}{M2} \times 1000$$

Where, Ml is the molecular weight of Alendronic acid D6.

M2 is the molecular weight of Alendronic acid D6 salt.

Provide an appropriate batch number and complete •^{S}IS stock Calculation and Dilution Preparation Sheet". Store the solution in refrigerator at 2°C-8°C.

Prepare stock dilution of internal standard in the concentration of 0.500 pg/mL using diluent as mentioned in the Table5

Prior to the processing of samples, 50 PL of internal standard is added to each sample.

Quality Control (QC) Samples

Prior to spiking, prepare stock dilutions of Alendronate in the concentration range as mentioned belowin Table 6.

Spiking of plasma for QC samples

Transfer 0.2 mL of the stock aliquot of corresponding concentrations of the above mentioned stock dilutions of Alendronate into 10 mL volumetric flask and make up to the volume with plasma to achieve the following concentrations as described in the **Table 7.**Aliquot 600 gL of each quality control samples into appropriately labeled and capped polypropylene tubes and store in ultra low temperature freezer at -70°C±15°C and low temperature freezer at -30°C ± IO°C for long term plasma stability and freeze-thaw stability.

III. RESULT

A selective and a sensitive LC-MS/MS method is used to determine and validate Alendronate in human plasma with the range of concentration 4.1386 ng/mL to 262.9557 ng/ml. The aim of our work is to develop a validated method for the determination of Alendronate in human plasma by using UPLC MS/MS. The Alendronic acid D6 as internal solution (IS). Alendronate was extracted by using liquid-liquid extraction technique achieved on Luna 3µ HILIC 200A (100 mm×2.0 mm column. The retention time was about 2.5 mins, high sensitivity and selectivity are required for any developed technique to control the allowed limits.Therefore, this study ensures that the peak ranges are calculated and the linear concentration ranges shows with optimized study results.Chromatographic representation refer in **Figure 2**and **3.**

Biological matrix

Blank K_2EDTA human plasma lots were used for screening.Table:8. The plasma samples are procured from Micro Therapeutic Research Labs Pvt. Limited, Chennai.

Calibration curve standard and quality control samples

Calibration curve standard and quality control all samples were prepared as per ICH guidelines and stored in -70 °± 15° C and -20°± 5° C.

METHOD VALIDATION AND CHARACTERISTICS

Selectivity and specificity

Six lots of blank plasma, one lot of heparin plasma, one lot of lipemic plasma, and one

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lot of hemolyzed plasma were tested for specificity and selectivity, demonstrating that the sample met the acceptance criteria and passed the test. Selectivity and Specificity are two terms that are often used interchangeably. The studied matrix ranged from mean value 0.1414, SD value 0.002473.(see Table 9)

Carry Over Test

Carry over is defined as the percentage peak area visible in a processed blank plasma injected in triplicate immediately after a processed ULOQ calibration standard derived from a PA batch sample. There was no substantial carryover for alendronate and as an internal standard.(see Table 10)

Precision and Accuracy

The assay's precision and accuracy were measured by the percentage co-efficient of variation over the concentration range of LOQQC, LQC, INTQC, MQC and HQC sample of Alendronate while processing methodvalidation.**Figure 3**.(see Table 11).

Linearity

Preparing 8 point standard calibration curve in human plasma is used to determine linearity concentration. Using Alendronate as an internal standard and a CC curve, the concentration of Alendronate varied from 4.1386 to 262.9557 ng/ml. using Alendronic acid D6 as IS and for CC curve refer in **Figure 4**

Recovery studies of Alendronate

Alendronate recovery was assessed by comparing the detector response of analyte extracted low, medium, and high-quality control samples from extracted CC and QC samples from PA batch with detector response from un-extracted aqueous samples at three levels. The average recovery rate for Alendronate was 82.20 percent. The % CV of Alendronate was 6.12 percent at three different QC levels. The average IS recovery for Alendronic acid D6 was 85.25 percent.

IV. DISCUSSION

A selective and sensitive LC-MS/MS method is used to determine and validate Alendronate in human plasma with the range of concentration 4.1386 ng/mL to 262.9557 ng/ml. The aim of our work is to develop a validated method for the determination of Alendronate in human plasma by using UPLC MS/MS. The Alendronic acid D6 is the internal solution (IS). Alendronate was extracted by using the liquid-liquid extraction technique achieved on Luna 3µ HILIC 200A (100 mm×2.0 mm column).The retention time was about 2.5 mins, high sensitivity and selectivity are required for any developed technique to control the allowed limits. Therefore, this study ensures that the peak ranges are calculated and the linear concentration ranges show with optimized study results.

V. CONCLUSION

The bio analytical method development was developed as per ICH guidelines, a selective and sensitivity LC-MS/MS method to quantify Alendronate in human plasma over the concentration range 4.1386 to 262.9557 ng/ml. It has been successfully validated in the experimental parameters and resulted values are shown in **Table 12.**The goal of this study was todevice a method for validating Alendronate levels in human plasma that was both sensitive and quick. As a result, the peak ranges show our method appear to be selective,sensitive,fast, and appropriate for clinical pharmacology in human.

Table 1: Drug profile.

PROFILE	ALENDRONATE	ALENDRONATE D6
IUPAC NAME	(4-amino-1-hydroxy-1-phosphonobutyl)phosphonic acid	4-Amino-1-hydroxybutane-1,1-diphosphonic Acid
CHEMICAL FORMULA	$C_4H_{13}NO_7P_2$	C4H7D6NO7P2
MOLECULAR WEIGHT	249.096 g/mol	249.10 g/ mol
SOLUBILITY	Water, Alcohol, chloroform	Water, alcohol

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PHYSICAL PROPERTIES	Appearance: White powder Colour :White, crystalline Odour: odour less	Appearance: White powder Colour : white, crystalline Odour: odour less
HALF LIFE	24 hr	24 hr

Table 2: LC-MS/MS condition.

Molecule	Parent (m/Z)	Daughter (m/Z)	Dwell (sec)	Cone (Volts)	Collision energy (v)
Alendronate Sodium	348.21	162.96	0.200	35.0	25.0
Alendronic acid D6	354.20	168.00	0.200	35.0	25.0

Table 3:Preparation of Alendronate stock dilutions.

Stock Concentration (pg/mL)	Stock aliquot (mL)	Diluent Added (mL)	Final Volume (mL)	Final Concentration (pg/mL)	Stock CCID
919.4481	0.040	3.960	4.000	9.1945	STD SS H
9.1945	1.600	0.400	2.000	7.3556	STD SS G
7.3556	1 .200	0.800	2.000	4.4134	STD SS F
4.4134	1 .200	0.800	2.000	2.6480	STD SS E
2.6480	1.200	0.800	2.000	1.5888	STD SS D
1.5888	1 .200	0.800	2.000	0.9533	STD SS C
0.9533	1.000	1.000	2.000	0.4767	STD SS B
0.4767	0.700	1.300	2.000	0.1668	STD SS A

Table 4: Preparation of spiked calibration curve standards.

Stock CCD	Stock Concentration	Stock Aliquot	Plasma Added	Final Volume mL	Final Concentration n mL	Spiked CCID
STD SS H	230.3480	200	9800	10.000	262.9557	STD H
STD SS G	184.2780	200	9800	10.000	199.2267	STD G
STD SS F	92.1400	200	9800	10.000	92.9338	STD F
STD SS E	55.2840	200	9800	10.000	51.1254	STDE
STD SS D	33.1700	200	9800	10.000	30.4539	STD D
STD SS C	19.9020	200	9800	10.000	18.2663	STD C
STD SS B	9.9520	200	9800	10.000	9.6352	STD B
STD SS A	3.9800	200	9800	10.000	4.1386	STD A

Table 5: Preparation of stock dilution.

Stock Conc. (gg/mL)	Stock Aliquot (mL)	Diluent Added (mL)	Final Volume (mL)	Final Concentration (µg/mL)
1000	0.020	1.980	2.000	10.000
10.000	I .250	23.750	25.000	0.500

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Table 6: Preparation of quality control(QC) samples.

Stock Concentration (ug /mL)	Stock Aliquot	Diluents Added	Final Volume (mL)	Final Concentration	Stock QCID
919.4481	0.032	3.968	4.000	7.3556	SS HQC
7.3556	l.200	0.800	2.000	4.4134	SS MQC
4.4134	0.700	1.300	2.000	1.5447	SS INTQC
1.5447	0.610	1.390	2,000	0.471 1	SS LQC
0.4700	0.720	1.280	2.000	0.1696	SS LOQQC

Table 7: Spiking of plasma for QC samples.

Stock QC ID	Stock Concentration (ug/mL)	Stock Aliquot	Plasma Added ILL)	Final Volume	Final Concentration (ng/mL)	Spiked QC ID
SS HQC	7.3556	200	9800	10.000	147.1 120	HQC
SS MQC	4.4134	200	9800	10.000	88.2680	MOC
SS INTQC	I .5447	200	9800	10.000	30.8940	INTQC
SS LQC	0.471 1	200	9800	10.000	9.4220	LQC
SSLOQQC	0.1696	200	9800	10.000	3.3920	LOQQC

Table 8: Loot details.

Supplier	Micro Therapeutics Research Labs Pvt Limited,chennai
Plasma Batch ID/Lot No.	MAT-18-0702-I MAT-19-0708-II MAT-19-0709-II MAT-19-0710-II MAT-19-0711-II MAT-19-0714-II MAT-19-0648-XII(H) MAT-19-0617-I(HEP))

Table 9: K2EDTA plasma screening for Alendronate and internal standard.

Plasma Lot ID	Specificity (Blank)		Selectivity (Spiked LLOQ)		% Interference in Blank		Area Ratio
	Analyte	IS peak	Analyte	IS peak	Analyte (<20%)	IS(<5%)	Analyte/IS
MAT-18-0702-I	54	0	2905	20352	1.8589	0.0000	0.1428
MAT-19-0708-II	26	0	2640	19255	0.9848	0.0000	0.1364
MAT-19-0709-II	0	0	3181	22497	0.0000	0.0000	0.1414
MAT-19-0710-II	0	0	4251	30293	0.0000	0.0000	0.1403
MAT-19-0711-II	0	0	3309	22961	0.0000	0.0000	0.1441
MAT-19-0714-II	0	0	2898	20441	0.0000	0.0000	0.1418
MAT-19-0648-XII(H)	10	0	2988	21466	0.3347	0.0000	0.1392
MAT-19-0617-I(HEP)	0	0	2702	18881	0.0000	0.0000	0.1431
				Mean	**0.39730**	**0.00000**	**0.14114**
						SD	**0.002473**
% of Lots passing =	**100**	**%**				**%CV**	**1.75**
						Result	**Pass**

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Table 10: Carry over test for Alendronate and Alendronic acid D6.

Sample ID	Analyte peak area	IS peak area
Extracted blank	8	4
Extracted LLOQ+IS	125	1120
Extracted ULOQ+IS	18545	15474
Extracted blank-I	7	5
Extracted blank-II	12	4
% Carry Over from Blank I	**-0.80**	**0.01**
% Carry Over from Blank II	**3.20**	**0.00**
Result for blank - I	Pass	Pass
Result for blank - II	Pass	Pass

Table 11: Precision and Accuracy study for Alendronate.

QC ID	LOQQC	LQC	MQC	HQC
Actual Concentration (ng/mL)	**4.0500**	**10.1260**	**91.3260**	**182.6520**
Calculated Concentrations (ng/mL)	4.256	9.558	98.8438	195.5681
	4.305	9.5727	96.5966	195.308
	3.4834	9.8248	99.4195	190.3836
	4.3536	9.9302	92.9415	179.3488
	4.4692	10.178	97.8712	193.6639
	4.4168	9.7358	99.559	192.6536
Mean	**4.21400**	**9.79992**	**97.53860**	**191.15433**
SD	**0.365941**	**0.234436**	**2.508173**	**6.086783**
%CV	**8.68**	**2.39**	**2.57**	**3.18**
%Nominal	**104.05**	**96.78**	**106.80**	**104.65**

Table 12: Summary of Experimental Parameters and Results of Validated LC-MS/MS Method for the Quantification of Alendronate in K2EDTA human plasma.

S.No	Experimental Parameters	Acceptable Range/Criteria (in %)	Result Obtained (in %)
1	Specificity and Selectivity	>80	100
2	Recovery	≤110%	Analyte -82.20%,IS-85.25%
3	Carry Over Test	Analyte <20 IS<5	Analyte - -0.80,IS- -0.01
4	Precision and Accuracy	80 ± 115%	LOQQC-104.05,LQC-96.78,MQC-106.80,HQC-104.65

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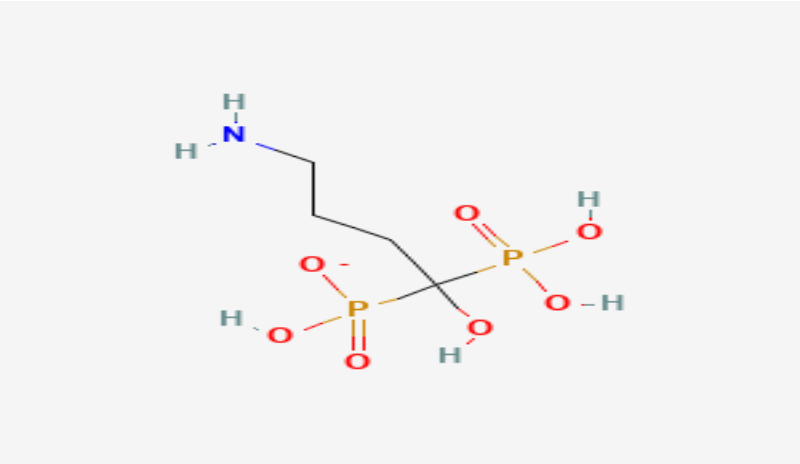

Figure 1: Chemical structure of Alendronate.

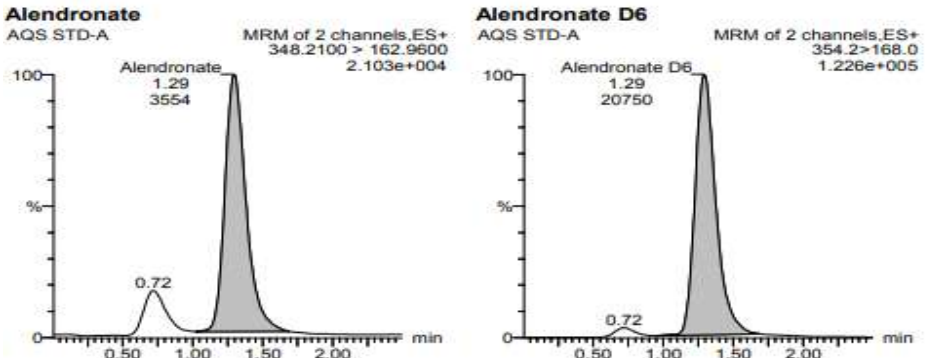

Figure 2: Representative chromatogram of standard A for Alendronate.

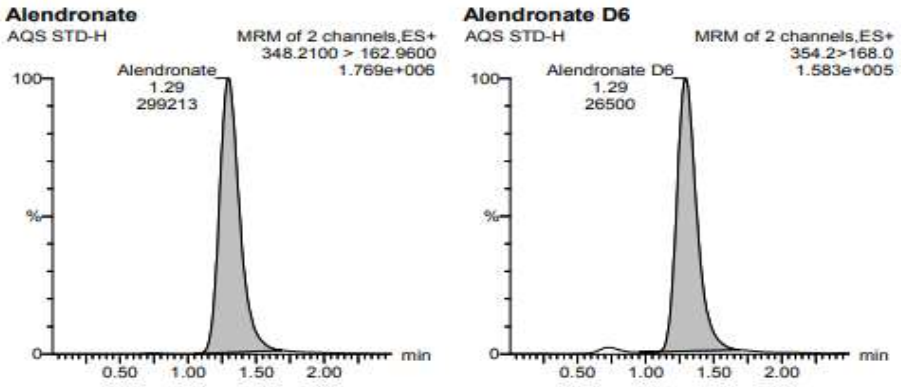

Figure 3: Representative chromatogram of standard H for Alendronate.

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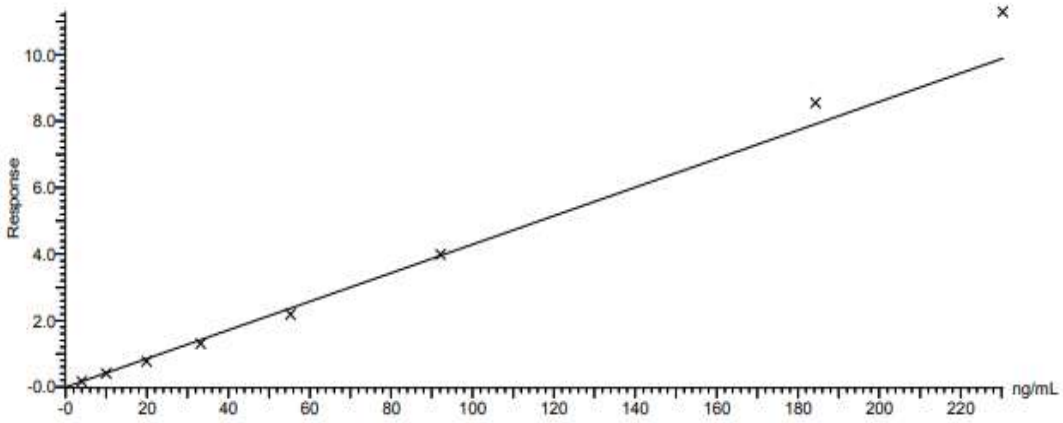

Figure 4: Calibration curve for Alendronte.

Compound name: Alendronate
Correlation coefficient: r = 0.995311, r^2 = 0.990645
Calibration curve: 0.0429633 * x + -0.00654299
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x^2, Axis trans: None

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS:

AR: Analytical Reagent
Amu : Atomic mass units
BA : Bio-Analytical
CC: Calibration Curve
GR: General Reagent
GL: Group leader
g : Gram
HQC: Higher Quality Control
UPLC : Ultra Performance Liquid Chromatography
INTQC: Intermediate Quality Control
KV: Kilo Volts
K_2EDTA : Di Potassium Ethylene Diamine Tetra Acetic Acid
LC-MS/MS: Liquid Chromatography-Mass Spectrometry/Mass spectrometry
LOQQC : Limit of Quantification Quality Control
LQC: Lower Quality Control
mg : Milligram
ml : Milliliter
mM: Millimolar
MQC : Middle-Quality control
MTR: Micro Therapeutic Research Labs Pvt. Ltd.
ng/ml : nanogram/milliliter
No : Number
PD : Positive Displacement
QA : Quality Assurance
QC : Quality Control
RIA : Radio Immuno Assay
RA : Research Associate
RS : Research Scientist.
rpm : Revolution per minute.
SOP: Standard Operating Procedure
TL : Team Leader
ULOQ: Upper Limit of Quantification
v/v : Volume by volume
TFA:Trifluro Acetic acid

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