SENSITIVE HPLC-MS/MS METHOD FOR ESTIMATION OF ELAGOLIX IN HUMAN PLASMA SAMPLES

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ABSTRACT

In the present work, a rapid, sensitive, specific, precise and accurate liquid chromatography-tandem mass spectrometry method for determination of Elagolix in human plasma was developed and validated with a large calibration curve range (10-4000 pg/mL) which can be used for routine drug analysis and bioequivalence studies. Liquid-liquid extraction method was used to extract the analyte from the human plasma. The separation was achieved using Xbridge Zorbax Eclipse XDB - C18 (150 x 4.6 mm, 5 µ) column with Acetonitrile: 20mM Ammonium formate (pH-3.0) (50: 50, v/v) as a mobile phase. A flow rate of 1.0 mL/min, no splitting and run time 10.0 min was used for the chromatographic analysis of Elagolix . Sensitivity of this method was found to be 10 pg/mL. The analyte was analyzed by mass spectrometry in the multiple reaction monitoring mode. A Turbo-Ion spray source was interfaced between the HPLC and triple quadrupole mass spectrometer (MDS Sciex API 4000). Where the acquired masses for Elagolix sodium 654.5 → 529.1 m/z and Elagolix-D6 was 638.4 → 529.1 m/z were used for quantification of an analyte and its IS. The method was validated in terms of accuracy, precision, selectivity, recovery, freeze-thaw stability, bench-top stability, stock solution stability and re-injection reproducibility. The within and between-batch precision was obtained within the range 0.31 to 8.55 and 0.26 to 6.16. The mean recovery for drug was obtained 87.79%, where as the mean recovery of IS was 84.97%. The %RSD value at higher concentration and lower concentration in all stability experiments was within 15%. This method is free from ion suppression, ion enhancement and any type of abnormal ionization.

Keywords: Elagolix, Deuterated, LC-MS/MS, Human plasma

INTRODUCTION

Elagolix sodium (Figure-1), the sodium salt of the active moiety elagolix. Elagolix sodium is a nonpeptide small molecule, GnRH receptor antagonist [1-5].

Elagolix sodium is chemically described as sodium 4-({(1R)-2-[5-(2-fluoro-3-methoxyphenyl)-3-{[2-fluoro-6-(trifluoromethyl)phenyl]methyl}-4-methyl-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl]-1-phenylethyl}amino)butanoate. Elagolix sodium has a molecular formula of C32H29F5N3O5Na and a

molecular weight of 653.58. Elagolix free acid has a molecular weight of 631.60. Elagolix sodium is a white to off white to light yellow powder and is freely soluble in water [6].

Elagolix sodium is a GnRH receptor antagonist that inhibits endogenous GnRH signaling by binding competitively to GnRH receptors in the pituitary gland. Administration of Elagolix results in dose-dependent suppression of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), leading to decreased blood concentrations of the ovarian sex hormones, estradiol and progesterone [3-7].

Sample pre-treatment has also been an item of interest for the analysis of Elagolix in biological samples. Previous methods included solid-phase extraction (SPE), protein precipitation and Liquid-liquid extraction (LLE). SPE is relatively expensive, time-consuming and complex for a large number of samples collected and treated. LLE with diethylether/hexane (80:20, v/v), methyl tert-butyl ether and diethyl ether has been reported, but hexane and methyl tert-butyl ether were toxic and expensive, where as diethyl ether were easy to evaporate.

The present investigation reports a simple a simple, sensitive, precise, economical and less toxic LC-MS/MS method for the analysis of Elagolix in plasma based on the LLE with ethyl acetate. The developed method was validated as per FDA guidelines [18-35].

MATERIAL AND METHODS

Instrumentation

The Agilent 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany) connected to the API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) with turbo electrospray interface in positive ionization mode was used. Data processing was performed on Analyst 1.4.1 software package (SCIEX).

Reagents / Materials

Elagolix sodium (EG) (Figure A) and Elagolix –D6 (EGD6) (Figure B) was obtained from Hetero Pharmaceuticals, Hyderabad, India. Water (HPLC Grade), Ammonium formate, Formic Acid (analytical grade) were purchased from Merck, Mumbai, India. Acetonitrile (HPLC Grade), Diethyl ether and dichloromethane (HPLC grade) were obtained from J.T. Baker, USA. Human plasma was procured from doctors lab pathologicals, Hyderabad. Milli Q water was taken from the in-house Milli-Q system.

Detection

Detection was done by turboionspray (API) positive mode with unit resolution. Quantification was by MRM, where the acquired masses for Elagolix sodium 654.5 → 529.1 m/z and Elagolix-D6 was 638.4 → 529.1 m/z (Figure.2 & 3).

Chromatographic conditions

Chromatographic separation was achieved with Acetonitrile: 20mM Ammonium formate (pH-3.0) (50: 50, v/v), gave the best peak shape and low baseline noise was observed using the Xbridge Zorbax Eclipse XDB - C18 (150 x 4.6 mm, 5 µ). The total analysis time was 10 min and flow rate was set to 1.0 mL/min. The temperature was set to 40°C for the column oven. The sample volume for the injection into mass spectrometry was adjusted to 10 µL for better ionization and chromatography. Elagolix sodium (EG) and Elagolix **(**EGD6) were eluted at 5.09 and 5.25 min, approximately, with a total run time of 10 min for each sample.

Preparation of standards and quality control samples

Standard stock solutions of EG (1.0 mg/mL) and EGD6 (1.0 mg/mL) were prepared in Methanol. The IS spiking solution (25.0 pg/mL) was prepared in mobile phase solution (Acetonitrile: 20mM Ammonium formate (pH-3.0) (50: 50, v/v) from EGD6 stock solution. Standard stock solutions and IS spiking solutions were stored in refrigerator conditions of 2–8ºC until analysis. Standard stock solutions of EG (1.0 mg/mL) were added to drug-free screened human plasma to obtain concentration levels of 10, 20, 50, 100, 400, 800, 1600, 2400, 3200 and 4000 pg/mL for analytical standards, and 10 (LLOQ), 30 (LQC), 1500 (MQC) and 3000 pg/mL (HQC) for quality control (QC) standards, and stored in the freezer at 30ºC until analysis. The aqueous standards were prepared in a mobile phase solution (Acetonitrile: 20mM Ammonium formate (pH-3.0) (50: 50, v/v) and stored in the refrigerator at 2–8ºC until analysis.

Biological Matrix

Human plasma containing K_2EDTA as anticoagulant was used as a biological matrix during method validation. Selectivity and sensitivity tests were performed before bulk spiking.

Sample preparation

The LLE method was used to isolate EG and EGD6 from human plasma. For this purpose, 50µL of EGD6 (10 pg/mL) and 100µL of plasma sample were added to the labelled polypropylene tubes and vortex briefly for about 5 min. Thereafter 3mL of extraction solvent (in the ratio of diethyl ether: dichloromethane, 50:50(v/v)) were added and vortex for about 10 min. Next, the samples were centrifuged at 4000 rpm for approximately 5 min at ambient temperature. From each, a supernatant sample was transferred into labelled polypropylene tubes and evaporated to a dryness of 40°C briefly, and then reconstituted with a mobile phase solution (Acetonitrile: 20mM Ammonium formate (pH-3.0) (50: 50, v/v), and the sample was transferred into autosampler vials and injected into the LC-MS for study.

Method Validation

The validation was performed as per FDA guidelines to evaluate the method in terms of linearity response, sensitivity, selectivity, precision and accuracy (within-batch and between-batch/inter-day), stabilities (freeze-thaw, bench top, short- term and long-term stock solutions, working solutions and long term stability in

matrix), carryover effects, recovery, dilution integrity, matrix effect, matrix factor, autosampler re-injection reproducibility and ruggedness experiment [9].

System suitability

System suitability experiment was performed by injecting six consecutive injections at least once in a day with using aqueous MQC solution. System performance experiment was performed by injecting sequence of injections at the beginning of analytical batch and % CV was calculated.

Selectivity and sensitivity

Selectivity was performed by analyzing human blank plasma samples from six different sources (donors) with an additional hemolyzed group and lipidemic group to test for interference at the retention times of analytes. The sensitivity was compared with the lower limit of quantification (LLOQ) of the analyte with its blank plasma sample. The peak area of blank samples should not be more than 20% of the mean peak area of the limit of quantification (LOQ) of EG and 5% of the mean peak area of EGD6.

Calibration of standard curve (Linearity and range)

The linearity of the method was determined by using standard plots associated with nine point standard curve including LLOQ and ULOQ. Concentration of calibration curve standards was calculated against the calibration curve and the linearity of the method was evaluated by ensuring the acceptance of precision and accuracy of calibration curve standards. Two consecutive calibration curve standards should not be beyond the acceptance criteria. The lower limit of quantification (LLOQ) was the lowest concentration at which the precision expressed by relative standard deviations (RSD, CV %) is better than 20% and the accuracy (bias) expressed by relative difference of the measured and true value was also lower than 20%.

Precision and accuracy

The within-run and between-run percentage mean of precision and accuracy of the EG were measured by the percent coefficient by using six replicate samples of variation over the concentration range of LLOQ (Lowe limit), LQC (Low), MQC (Middle) and HQC (high) quality control samples for the three precision and accuracy batches to their nominal values. The acceptable %coefficient of precision and accuracy should be less than 15%. The between and within batch % mean accuracy for LQC, MQC and HQC samples were within the range of 85.00-115.00% and for the LLOQ within the range of 80.00-120.00% respectively.

Recovery

The % mean recoveries was determined by comparing the mean peak area of the 6 replicates of extracted plasma quality control samples at high, middle 1&2 and low concentrations against respective mean peak area of the six replicates of un-extracted quality control samples at high, middle and low concentrations. A recovery of more than 50% was considered adequate to obtain required sensitivity. The % mean internal standard recovery was determined by comparing the mean peak area of internal standard in the extracted plasma quality control samples at MQC concentration against the mean peak area of internal standard in the un-extracted quality control samples at MQC concentration.

Ruggedness

Ruggedness of the method was evaluated by using different analyst and different column of the same make and model or different equipment of the same make and model. The ruggedness experiment should meet the acceptance criteria for linearity and intra-batch accuracy & precision.

Matrix effect

To predict the variability of matrix effects in samples from individual subjects, matrix effect was quantified by determining the matrix factor, which was calculated as follows:

$$\text{Matrix Factor} = \frac{\text{Peak response ratio in presence of extracted matrix (post extracted)}}{\text{Peak response ratio in aqueous standards}}$$

Six lots of blank biological matrices were extracted each in triplicates and post spiked with the aqueous standard at the mid QC level, and compared with aqueous standards of same concentration. The overall precision of the matrix factor is expressed as coefficient of variation (CV %) and % CV should be < 15%.

Stability of Elagolix sodium (EG) and Elagolix-D6 (EGD6)

Long term stock solution stability

Long term stock solution stability for EG and EGD6 (internal standard) were performed at the stock concentration by using six consecutive injections of aqueous standard equivalent to ULOQ concentration and working concentration respectively after storage of at least 4 days in the refrigerator at 2-8°C. Stability was assessed by comparing the stock dilutions of EG and EGD6 (internal standard) prepared from the freshly prepared stock solutions (comparison) against stock dilutions of EG and EGD6 (internal standard) prepared from the stock solutions stored at 2-8°C (stability). Long term stock solution stability was evaluated by comparing the mean response of stability samples against mean response ratios of comparison samples.

Stability of Drug in Biological Matrix

Perform the matrix stability experiment by using freshly prepared calibration curve standard and three replicates of freshly prepared batch qualifying quality control samples at HQC and LQC levels. The precision and accuracy for the stability samples must be within ≤15 and ±15%, respectively, of their nominal concentrations. Stability studies in biological matrix were conducted in the various conditions at LQC and HQC levels as described below:

Freeze thaw stability

Freeze thaw stability of the spiked quality control samples were determined after 1st and 3rd freeze thaw cycles stored at -20±5°C. Six replicates of each HQC and LQC samples were used for assessing each freeze thaw experiment (for first and third cycle at both the freezing temperatures). The first freeze-thaw cycle was of at least 24 hours followed by minimum of 12 hours for subsequent cycles. Process and analyze freeze thaw stability samples along with freshly spiked calibration curve and comparison samples (6 replicates of each LQC and HQC) in screened biological matrix. Evaluate the freeze thaw stability on the basis of % change of LQC and HQC samples. The % Accuracy and % CV of LQC and HQC should be within ± 15.00 and ≤ 15.00 respectively.

Bench top stability

Spiked quality controlled samples (6 replicates of each LQC and HQC) were stored in deep freezer at temperature -20±5°C, which was retrieved after minimum 12 hours of freezing and was kept at ambient temperature on working bench for recommended period of at least 21 hours. Six replicates of each HQC and LQC samples were used for assessing the bench top stability experiment. Upon the completion of recommended period, process and analyze bench top stability samples along with freshly spiked calibration curve and comparison samples (6 replicates of each LQC and HQC) in screened biological matrix. Evaluate the bench top stability on the basis of % Accuracy and % CV of LQC and HQC samples.

Autosampler re-injection reproducibility

Autosampler re-injection reproducibility was evaluated by re-injecting accepted precision & accuracy batch, which were stored preferably in either autosampler or in refrigerator for at least 71 hours or as per requirement. Autosampler re-injection reproducibility was evaluated by % Accuracy and % CV of LQC and HQC samples.

Long Term Stability in Biological Matrix

The long-term stability samples of LQC, MQC and HQC samples were kept frozen in vials at -20±5^{0}C for 99 days were assessed along with freshly processed calibration and comparison samples (six samples each of LQC, MQC, HQC). The initial EG concentration freshly after sample treatment preparation was assumed to be 100%. The selection of the stability duration is on the basis of the characteristic of the analyte(s).

RESULTS & DISCUSSION

Method Validation

System Suitability

System performance experiment was performed by injecting six consecutive injections at the beginning of analytical batch. % CV was 1.35.

Carryover Test

For carryover test two samples of upper limit of quantification (ULOQ) and 4 samples of blank plasma were processed. These samples were injected in the following sequence.

a) 2 blank samples b) 2 ULOQ samples c) 2 blank samples.

The step (b) and (c) were repeated 2 times. The results demonstrate that there was no interference from the previous injection.

Selectivity and specificity

The analysis of EG and EGD6 using MRM (Multiple reaction monitoring) function was highly selective with no interfering compounds. Chromatograms obtained from plasma spiked with EG and EGD6 represented in Figure. 3.0.

Limit of Detection (LOD) and Quantification (LOQ)

The limit of detection was used to determine the instrument detection levels for EG even at low concentrations. 30 µL of a 1.0 pg/mL solution was injected to give an on-column mass of 0.03 pg/ml. The limit of quantification for this method was proved as lowest concentration of the calibration curve which was proved as 10.0 pg/ml.

Calibration curve standards, Precision and Accuracy

Calibration curves were plotted as the peak area ratio (EG/EGD6) versus (EG) concentration. Calibration was found to be linear over the concentration range of 10.0- 4000.0 pg/mL. The determination coefficients (r^2) were greater than 0.9997 for all curves (Figure.2 & Table. 1). As shown in Table-1 the intra-batch CV% was 0.71 to 8.55 and inter-batch CV% was 0.26 to 6.16%. These results indicate the adequate reliability and reproducibility of this method within the analytical range.

Recovery

The recovery following the sample preparation using Liquid-liquid extraction with Methyl tertiary butyl ether was calculated by comparing the peak area of EG in plasma samples with the peak area of solvent samples and was estimated at control levels of EG. The overall average recovery EG and EGD6 were found to be 87.79 and 84.97% respectively (Table. 1).

Matrix effect

Six lots of blank biological matrices were extracted each in triplicates and post spiked with the aqueous standard at the mid QC level, and compared with neat standards of same concentration in alternate injections. The overall precision of the matrix factor is 5.11 for EG. There was no ion- suppression and ion-Enhancement effect observed due to IS and analyte at respective retention time.

Stock Solution Stability

Stock solution stability at refrigerator (2-8°C)

Stock solution each of EG and internal standard were stable after approximately 56 days and at refrigerated temperature 2-8°C. For EG and EGD6 (internal standard) the %Accuracy was 91.65 and 87.81 respectively.

Stability of EG in plasma samples (Freeze - thaw, Auto sampler, Bench top, Long term)

Quantification of the EG in plasma subjected to 3 freeze-thaw (-30^{o}C to room temperature) cycles show the stability of the analyte and %CV was between 1.31 to 1.79. No significant degradation of the EG was observed even after 71 hr storage period in the autosampler tray and the %CV was between 1.1 to 1.7. No significant degradation of the EG was observed even after 21 hr storage period in the room temperature and %CV was between 1.2 to 1.5 of the theoretical values. In addition, the long-term stability of EG in QC samples after 99 days of storage at -20^{o}C was also evaluated and %CV was ranged from 0.25 to 1.28. These results confirmed the stability of EG in human plasma for at least 99 days at -20^{o}C (Table-1).

CONCLUSION

The LC–MS/MS validated method has proved to be very simple, sensitive and reliable. The assay method is specific due to the inherent selectivity of tandem mass spectrometry. The major advantage of this method is the use of deuterated Elagolix-D6 as an internal standard. The run time is within 8 min and only 0.200 mL of plasma was required for each determination of Elagolix, and thus the stress to volunteers or patients in clinical studies was greatly reduced. This method is very suitable and convenient for pharmacokinetics and bioavailability study of the drug Elagolix.

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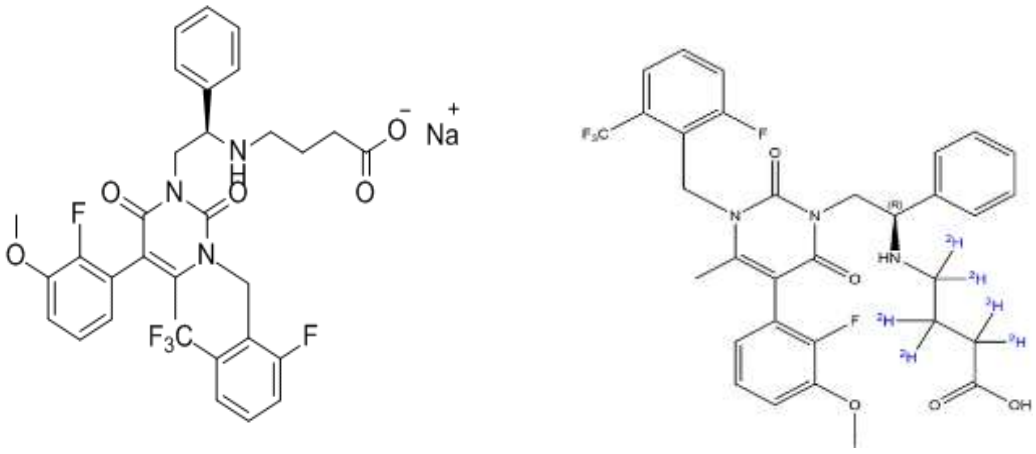
TABELS

Table.1.0: Validation parameters

Analyte	Elagolix sodium
Internal Standard (IS)	Elagolix D6
Method description	Liquid-liquid extraction with LC-MS/MS technique
Selectivity	No known, endogenous plasma components, common drugs and commonly used female contraceptives interfere with the analytical assay
Limit of quantitation	10.0 pg/mL
Limit of Detection	0.03 pg/ml
Average recovery of drug	87.79 %
Average recovery of IS	84.97 %
Standard curve Linearity & Regression Regression model Analysis method	10.0 to 4000.0 pg/mL & 0.9997 Weighted ($1/conc^2$) linear Peak area ratio (PAR)
QC concentrations	QC A: 30.00 pg/mL QC B: 1500.00 pg/mL QC C: 3000.00 pg/mL
QC Intraday precision range	0.31 to 8.55%
QC Inter day accuracy range	0.26 to 4.52%
Matrix factor	5.11
Bench-top stability (Room temperature) & %CV	21 hours & 1.2 to 1.5
Processed stability (Rrefrigerated condition) & %CV	71 hours & 1.1 to 1.7%
Freeze-thaw stability (-20°C) & %CV	3 cycles & 1.29 to 1.79%
Long-term storage stability (-20°C) & %CV	99 days & 0.25 to 1.28
Stock solution stability & %Accuracy *Analyte (Room temperature)* *Internal standard (Refrigerated condition)*	 9.5 Hrs and 91.63% 56 days and 100.43%

FIGURES



A **B**

Fig. 1.0: Chemical Structure A) Elagolix Sodium (EG) B) Elagolix-D6 (EGD6)

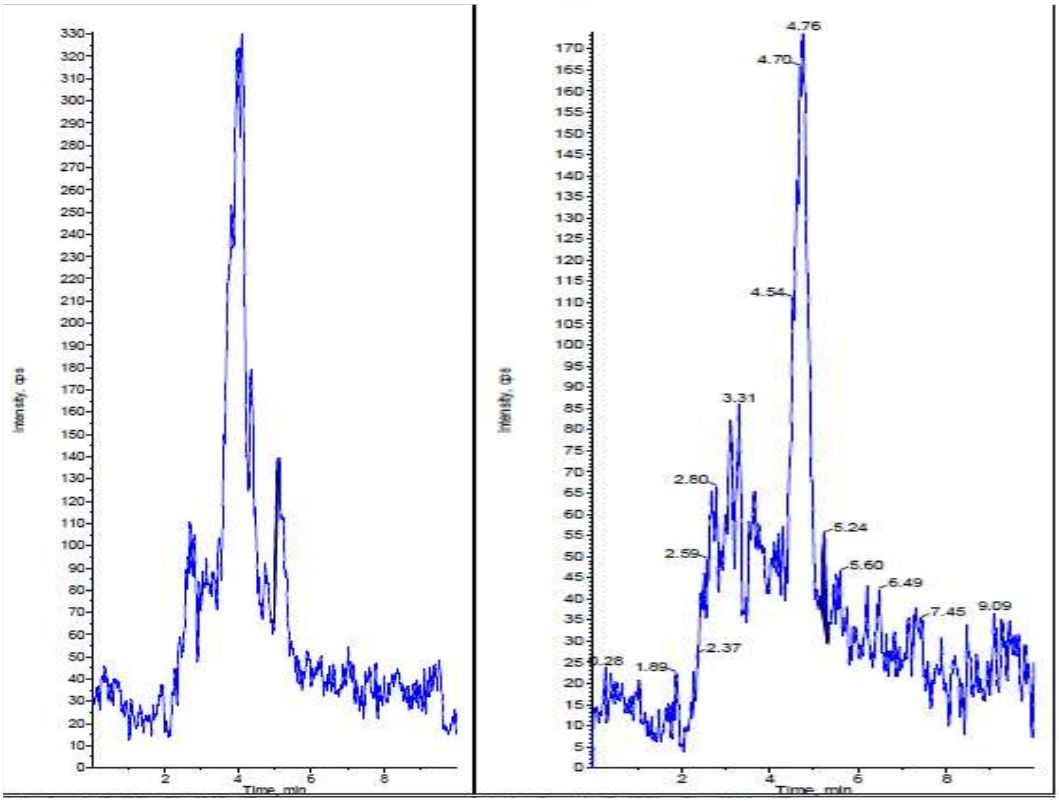
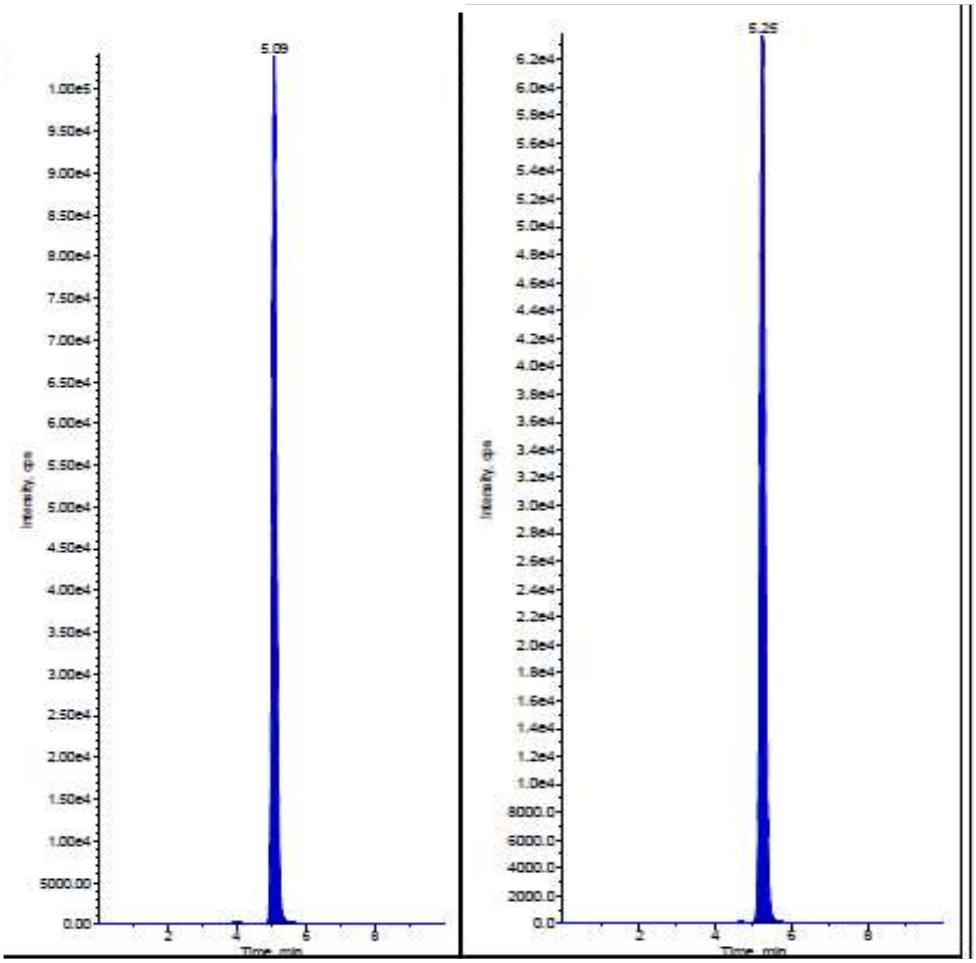


Figure.2.0: Blank plasma chromatogram of interference free Elagolix sodium (EG) and Elagolix-D6 (EGD6)



A **B**

Fig.3.0: Standard Chromatogram of Elagolix sodium (EG) and Elagolix-D6 (EGD6)

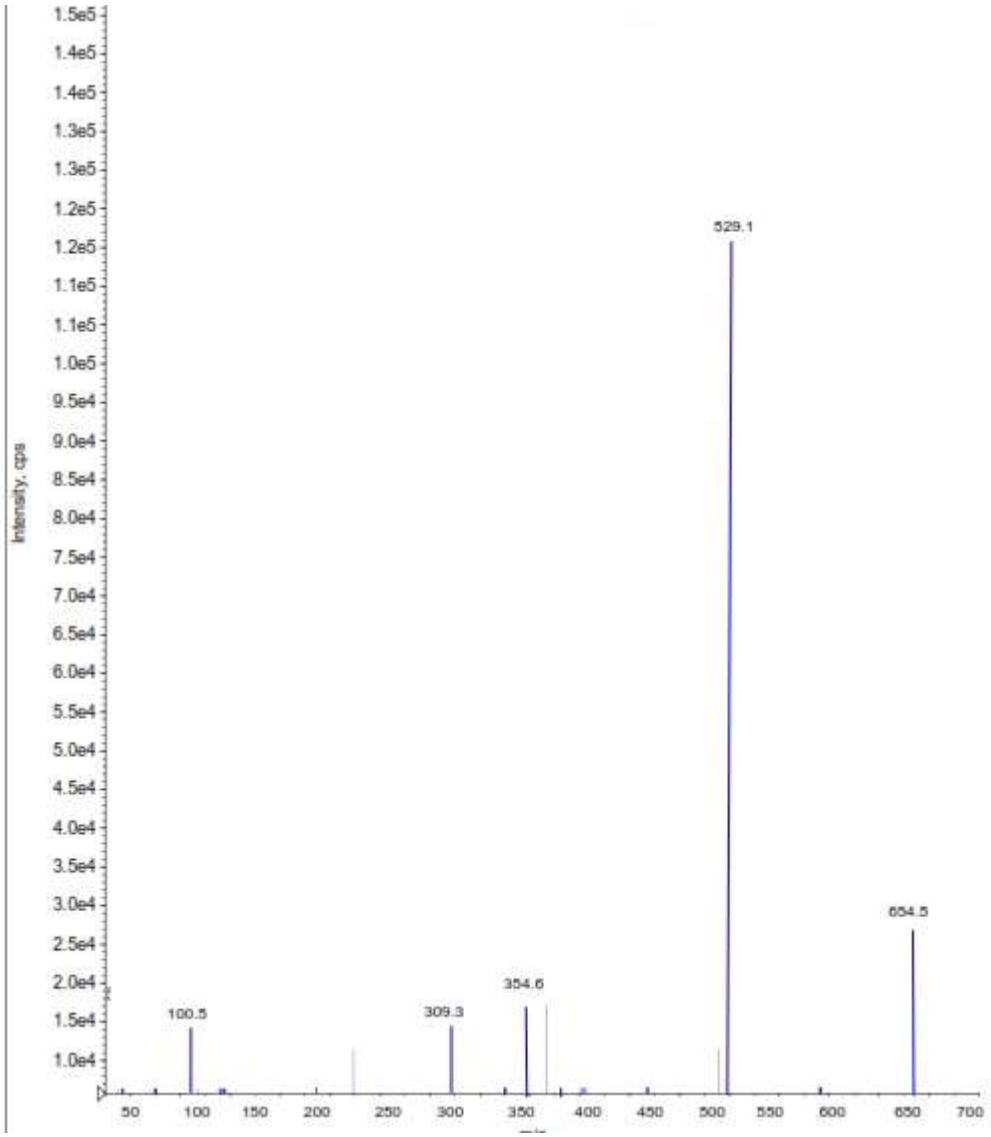


Figure.4.0. Mass scan spectrum of Elagolix sodium

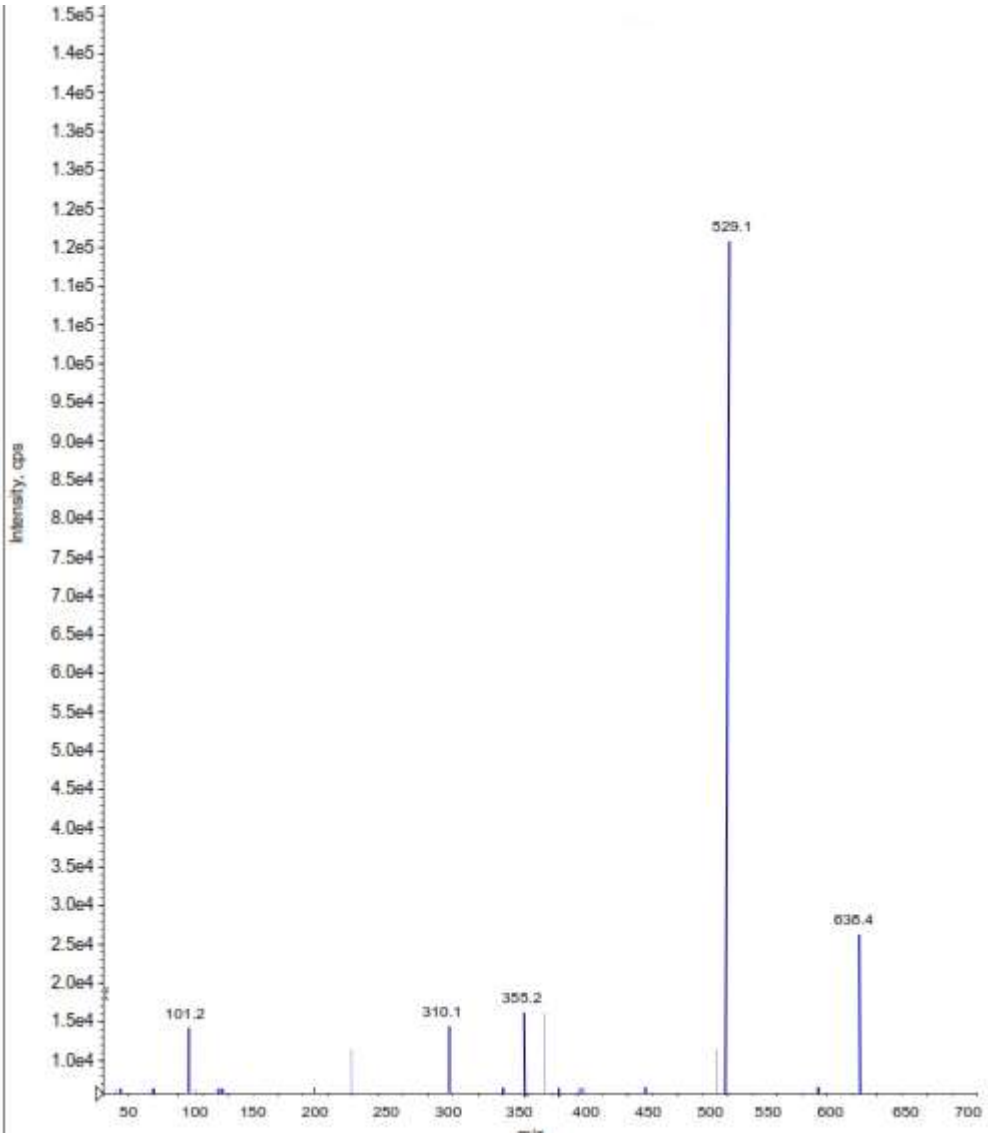


Figure.5.0. Mass scan spectrum of Elagolix-D6