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A Sensitive LC-ESI-MS/MS Method to Quantify Tibolone as Oxime-Derivative in Human Plasma and its Application to a Pharmacokinetic Study

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ABSTRACT

A novel, sensitive and reproducible liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed and validated for the estimation of tibolone ensuring a proper resolution from its isobaric metabolite Δ-tibolone in human plasma. The isotopic labeled Δ-tibolone D6 was used as the internal standard. Chromatography separation was done on Zorbax XDB C_{18} column and resolution was achieved by a gradient separation with a total run time of 8.50 minutes. The detection using Waters XEVO–TQ–S mass spectrometry system with a mass transition ion-pair of *m/z* 328.2 → 295.2 for tibolone and 334.2 →129.0 for δ-tibolone D6. Tibolone method involves derivatization to for its oxime and further sample cleanup was done by solid phase extraction technique. The method has been established for a linear range of 10.178–2016.040pg/ml with a correlation coefficient ≥0.99. The precision (% RSD) was less than 5.70% and accuracy (% RE) was within ±6.1%. The overall recoveries for tibolone and Δ-tibolone D6 were 72.88% and 99.28% respectively. The method was successfully applied for quantification of tibolone as a supportive data for a pharmacokinetic study of tibolone by following a single oral dose of tibolone (2.5mg) tablet in 18 healthy postmenopausal female human volunteers under fasting condition.

1. INTRODUCTION

Tibolone is a synthetic steroid an analog of progestin is used mainly in hormone replacement therapy [HRT] in postmenopausal women [1], it has also been evaluated in treatment for female sexual dysfunction [2] and endometriosis [3]. The estrogenic effect on bone also suggests using to for delaying osteoporosis as a part of HRT and plays the significant role in alleviating the hot flushes [4–5].

Tibolone is a 19-nor-testosterone derivative and is chemically identified as 17-Hydroxy-α-methyl-19-nor-17α-pregn-5(10)-en-20-yn-3-one[6]. Tibolone has a complicated drug metabolism and with varied pharmacology. Tibolone and Δ-tibolone which is a pharmacologically inactive metabolite are the agonists of the progesterone and androgen receptors[7]. Tibolone has two major active metabolites 3α hydroxytibolone and 3-ß hydroxytibolone which are agonist to estrogen receptor, wherein 3α hydroxytibolone being more active as compared to 3ß hydroxytibolone[8].

The use of HRT has been drastically increased globally the need for monitoring the side effects and pharmacokinetic behavior as an individual drug and co-administered drug is gaining high importance[9].

Tibolone undergoes bioconversion in the intestine and liver to its androgenic metabolite, Δ-tibolone and estrogenic metabolites 3α-hydroxy tibolone & 3ß-hydroxy tibolone. Tibolone and Δ-tibolone bind to progestogenic receptors (PR) [10-11]. Though Tibolone is not considered for evaluation of bioequivalence, the concentrations are reported as a supportive data with maximum concentration (C_{max} & T_{max}), whereas the bioequivalence is mainly evaluated on one of its major metabolite 3α-hydroxy tibolone.

Independent method for quantification of tibolone metabolites has been developed and due to the complexity in the chemical nature, a single method cannot be established for tibolone and all its metabolite. There are few publications for determination of 3α-hydroxy tibolone and 3ß-hydroxy tibolone [12–14]. Till date, there is no published analytical method with LC/MS technique for determination of tibolone in human plasma.

The major aim of the present work was to develop a high throughput and sensitive bioanalytical method to quantify tibolone in support for analysis in the healthy human study.

Structurally as tibolone is similar to most of the steroid hormones consisting of three six carbon rings and 1 five-carbon ring, arranged in 6,6,6,5 patterns. Tibolone is structurally similar to other oxosteroid hormones like testosterone and progesterone, which are known to have low ionization efficiency, and is comparatively low and the hydrophobic nature. Another important challenge was to have a method, which also exhibits a proper resolution of its isobaric metabolite Δ-tibolone, which does not have any clinical significance and need not be quantified but should be well resolved from the parent compound Tibolone.

The keto (>C=O) functional group can be derivatized by oximation reaction thereby enhancing derivatives are used in oxosteroid estimation [15-17]. To date, for quantification of tibolone with chemical derivatization has not been published and the use of delta tibolone D6 as the internal standard with a different retention time indirectly proves that isobaric metabolite resolution.

The current study aims to develop an ESI–LC–MS/MS method for the quantification of tibolone in human plasma by chemical derivatization using hydroxylamine to enhance sensitivity, signal-to-noise (S/N) and throughput with the lowest limit of quantification (LLOQ) 10pg/mL and apply for bioequivalence study in human volunteers.

2. EXPERIMENTAL

2.1 Chemicals and reagents

The working standards of tibolone and Δ-tibolone D6 were purchased from Clearsynth (Mumbai, India). High purity water was obtained from a Millipore water purification system (Bangalore, India). Gradient grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). LC/MS grade formic acid was purchased from fisher scientific (Geel, Belgium) and hydroxylamine hydrochloride (AR grade) was purchased from Spectrochem, India. 1cc SPE cartridges of HLB were purchased from Water, USA limited. Drug-free (blank) human plasma was obtained from Drug Monitoring Research Institute (Mumbai, India) and was stored at -20°C prior to use.

2.2. Preparation of working solutions of calibration standards and quality control samples

Stock solutions of tibolone and internal standard (IS) Δ-tibolone D6 were prepared by dissolving in methanol to obtain a concentration of 1mg/mL. Tibolone was subsequently diluted using 50% methanol solution to obtain a series of working concentration of 1, 2, 8, 23, 46, 92, 122, 164, 200 ng/mL for a nine-point calibration curve. The working solutions of quality control samples were prepared using an independent stock at concentrations of 1, 2.7, 89, 160 ng/mL. The internal standard dilution of 1.5ng/mL was used during sample preparation. All solutions were stored at 2-8ºC until use. Plasma samples of calibration curve & quality control samples for tibolone were prepared by spiking 2% of tibolone of the working dilutions in interference-free lank plasma to obtain a final concentration of 2000, 1600, 1200, 900, 460, 230, 80, 20 and 10pg/mL and QC samples were also prepared similarly at concentration of 1600, 900, 2 and 10pg/mL respectively.

Final concentrations with actual weighing corrections ranged from 10.178 to 2016.040pg/ml and Quality control samples at four concentration levels were 10.342, 27.953, 901.699 and 1595.927pg/ml respectively.

2.3. Sample Extraction and Derivatization

After sample thawing an aliquot of 600µl of plasma sample was mixed with 50 µl of internal standard working solution (0.1 µg/ml of Δ-tibolone D6) and 300µL of 0.1% formic acid solution was added and vortexed. The sample mixture was loaded into an Oasis HLB (1 cm^3/30 mg), extraction cartridge which was pre-conditioned with 1 ml methanol and 1 ml water. The extraction cartridge was washed with 2 ml water. Tibolone and Δ-tibolone D6 were eluted with 0.3 ml of methanol. An equal volume of 1% Hydroxylamine was added to the sample and incubated at 60ºC for 30 minutes. The reaction was terminated by addition of 0.4mL of 0.1% formic acid in methanol was added to terminate the derivatization reaction. The sample was evaporated to dryness and reconstituted with 300µL of reconstitution solution, the samples were transferred to conical HPLC vial, and 15µl was injected into the UPLC–ESI-MS system.

2.4. Instrumentation

UPLC separation was carried out on Waters UPLC comprising of the binary pump, column oven, autosampler aligned with Waters XEVO–TQ-S mass spectrometer, a triple stage quadrupole mass analyzer with photomultiplier detector, equipped with electrospray ionization (ESI) source with a step-wave technology(Waters Ltd., UK). Mass Lynx 4.1 software was used acquisition and analysis. The liquid chromatography separation was performed on a Zorbax XDB C_{18} (150 x 4.6 mm, 3µm particle size) at 40°C. A mobile phase consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B) at a flow rate of 0.6 ml/min with a time and solvent gradient composition. Initial gradient at 50% 'A' followed by a linear gradient to 75% 'A' over 4.0 min, held at 90% 'A' with a linear flow gradient from 0.6ml/min to 0.9ml/min up to 7.5 min, shifted to initial gradient of 50% 'A' up to 8.0 min and then held constant until the end of the run for column equilibration. The total run time was 8.5 min and sample injection volume was 15µl. The mass spectrometer was operated in the multiple reaction–monitoring (MRM) mode. Sample introduction and ionization was ESI in the positive ion mode. The MS parameters for analytes are listed in **Table 1**.

2.5. Validation

The developed method was validated in accordance with the Guidance to the Industry-Bioanalytical Method Validation recommended by USFDA and EMEA guidelines [18-19].

The Specificity was evaluated by analyzing the blank plasma samples from eight different sources including lipemic and hemolyzed plasma. Samples were compared with the response of 10 pg/mL concentration of tibolone to test no interference at the retention time of tibolone and internal standard Δ-tibolone D6. Selectivity was evaluated in the presence of the 3α hydroxy tibolone, 3ß hydroxy tibolone, and Δ-tibolone and most commonly used over-the-counter (OTC) like ranitidine, paracetamol, ibuprofen and aspirin. Sensitivity was determined by analyzing six replicates of blank human plasma spiked with the analyte at the lowest level of the calibration curve.

Linearity was assessed by nine-point calibration curve from a range of 10.113–2029.388pg/mL in human plasma in four analytical runs acquired in different days. The curves were constructed by plotting the peak area ratio of tibolone to the internal standard versus nominal concentration of tibolone.

The intra–run and inter-run accuracy were determined by replicate (n=6) analysis of quality control samples and at LOQ that were extracted from the sample batch. The impact of co-administered drug ethinyl estradiol on the determination of tibolone was evaluated by performing precision and accuracy (n=6) was determined at lowest limit of quantification, low, middle and high levels.

Accuracy is defined as the percent of relative error ($\%RE$) and was calculated using the formula $\%RE = (E - T) \times (100/T)$, where E is the experimentally determined concentration and T is the theoretical concentration. Assay precision was calculated by using the formula $\%RSD = (SD/M) \times 100$, where $\%RSD$ is the coefficient of variance, M is the mean of experimentally determined concentrations and SD is the standard deviation.

The extraction efficiencies of tibolone and Δ-tibolone D6 were determined by comparing the peak area of extracted analytes to the peak area of non-extracted derivatized standards (analyte spiked post extraction in blank plasma).

The stability of tibolone was evaluated under various storage and handling conditions using low, middle and high QC samples. The stability QC samples were compared with freshly prepared QC samples (time 0). The stability was evaluated by comparing the mean back-calculated concentrations of stability samples with that of freshly prepared QC samples.

Freeze-thaw stability was evaluated for five complete cycles at –75ºC to room temperature. Short–term stability in plasma was evaluated by keeping the sample at room temperature for more than 6hrs. In–injector stability was evaluated by re-injecting the samples kept in autosampler (at 10ºC) for specific hours. Long-term stability of spiked human plasma samples for tibolone is to be established.

Stability was determined by calculating the % change and was calculated using the formula $\%Change = (S - C) \times (100/C)$, where S is the mean stability sample concentration and C is the mean of freshly prepared or comparison sample concentration. Analytes were considered stable if the $\%Change$ was within ±15% of freshly prepared or comparison sample concentration.

Matrix effect was evaluated with eight different lots of female plasma containing K_2 EDTA as anticoagulant including hemolyzed and lipemic plasma lot. Blank samples were prepared from each lot and were post–spiked with aqueous (derivatized) samples for tibolone and Δ-

tibolone D6 at low, middle and high QC levels. The post-spiked samples along with six replicate injections of aqueous spiked samples of LQC, MQC, and HQC were analyzed. The matrix effect was evaluated by calculating the matrix factor for tibolone and Δ-tibolone D6, internal standard normalized matrix factor for tibolone and matrix factor for mean ratio of tibolone and Δ-tibolone D6.

Matrix effect was performed with the aim to see the variability of plasma between different lots on the % RSD for mean area response and area ratio. It is considered there was no matrix effect if the % RSD for a mean area and mean response ratio was less than 15%.

3.0 RESULTS AND DISCUSSION

3.1. MS/MS & UPLC conditions optimization

Tibolone, an oxosteroid has moderate ionization efficiency, the formation of a protonated ion [M+H^+] would not be sufficient to be detected at lower concentrations. A solution of about 50 ng/mL was infused using the in-built infusion pump and the tuning was done with the underivatized sample wherein the signal of the protonated ions was observed with a moderate response. The ionization was also verified in the negative mode and with APCI source; there was no significant enhancement in either of the ionization modes. The ionization was acceptable but to increase the sensitivity and detect further lower concentrations the ionization capacity was to be increased. Considering the drug being used in female population the use of low sample volume was equally important.

3.2. Derivatization

As discussed by *Griffith et al.* [16], ionization enhancement by simple derivatization using hydroxylamine hydrochloride, wherein the carboxyl functional group is substituted with N−OH moiety was considered for tibolone. The formation of the tibolone–oxime (T−ox) derivative has significantly enhanced the ionization. The oxime reaction for the >C=O has been earlier developed at our lab for Gestodene [20] & Levonorgestrel [21], therefore only minor changes specific to tibolone were optimized for the concentration, temperature and time for a complete derivatization. The concentration of about 1% hydroxylamine at 60°C and for the duration of 30 minutes has shown complete and uniform derivatization, which was confirmed by the absence of underivatized compound in the sample.

The compound and source parameters have been optimized, during tuning of the compound two major product ions (m/z) of 313.1 and 295.2 were found to have a profoundly significant response for tibolone. Among the identified product m/z of 295.2 was found to have a consistent and reproducible signal. The final MRM parameters of the Tibolone oxime and Δ-tibolone D6 were 328.2/395.2 and 334.2/129.1 respectively. The selected fragments of parent and product ion for tibolone and its internal standard to be monitored are indicated in Figure-1 and Figure-2.

The chromatography optimization to achieve proper resolutions mobile phases was evaluated at different pH ranging from acidic to basic were tried, the optimal peak symmetry, low baseline noise with good signal to noise (S/N) was achieved at acidic conditions of a pH about 3.0±0.1. The mobile phase of acetonitrile-Pump-A and 0.1% Formic acid -Pump-B with gradient flow has shown the best chromatographic separation.

Columns including C_8, $C_{18,}$ phenyl-hexyl, and Cyano make, with varied column lengths ranging from 50 mm to 150 mm with a porosity of 5µm, 3µm and sub-2 micron columns were tried and the best results could be achieved in Zorbax XDB C_{18} (150 x 4.6 mm, 5µ) column. Pre-column chemical derivatization has significantly enhanced the S/N ratio with good peak symmetry with a low peak width and shorter runtime.

The involvement of derivatization using hydroxylamine in the sample extraction protocol has added the requirement for better sample cleanup, hence traditional sample cleanup of precipitation and liquid-liquid extraction were not tried. Solid Phase Extraction trials with generic protocol were tried but there was significant interference and low recovery along with significant matrix suppression was observed in the sample. The initial sample cleanup to remove the possible phospholipids was done by SPE using hydrophilic–lipophilic balance (HLB) cartridges. Post derivatization samples contained the excess hydroxylamine; hence, an additional sample cleanup using mixed mode cation exchange (MCX) cartridges has been considered thereby ensuring complete removal of the traces of hydroxylamine from the sample. This extraction methodology had ensured better Signal-to-Noise (S/N), sensitivity, recovery with no matrix effect and interference being observed at the retention time of tibolone and Δ-tibolone D6.

3.3. Specificity & Selectivity

Utilization of predominant product ions for each compound enhanced mass spectrometric specificity. The mass transition ion-pair selected were 328.2 → 295.2 for tibolone and 334.2 → 129.0 for Δ-tibolone D6 and these transitions were found to be specific for both the analytes.

Chromatographic specificity of the method was demonstrated by the absence of endogenous interfering peaks at the retention times of tibolone and Δ-tibolone D6 in eight different lots of extracted blank plasma. Selectivity of the derivatized moieties has been demonstrated for tibolone and Δ-tibolone D6 wherein there was no significant response at the retention time of each compound individually and in the presence of concomitant drugs. Representative chromatograms of extracted blank plasma and extracted plasma samples containing 10.113pg/ml tibolone (LLOQ) are presented as Figure-3 & Figure-4 respectively.

3.4. Selectivity

Method selectivity for tibolone in the presence its metabolites, internal standard and their impact on the quantification of tibolone was evaluated. A Blank sample, a blank with the internal standard sample and blank samples spiked with 3α & 3ß hydroxyl metabolites at a concentration of about 1500pg/ml concentration of each drug. Similarly, a blank sample spiked with only Δ-tibolone at a concentration of 1500pg/mL was also prepared in duplicate. The plasma samples were then processed and analyzed to investigate possible interference. No interference was observed at the retention time of Tibolone and Δ-Tibolone D6 in analyzed samples. There was no additive response observed in the ULOQ sample in the presence of all the metabolites. The selectivity evaluated for Tibolone and its internal standard MRM and found acceptable. Representative chromatogram of extracted blank in the presence of 3α hydroxyl tibolone & 3ß hydroxyl tibolone and isobaric metabolite delta-tibolone are presented in Figure-5. The results are represented in the Table 2 which confirm the selectivity of the analytical method.

3.5. Linearity

Linearity was assessed by analyzing four calibration curves in human plasma at nine levels on different days. The peak area ratios (area of tibolone/area of Δ-tibolone D6) of calibration standards were proportional to the concentration of analytes in each assay over the nominal

concentration range of 10.178–2016.040pg/ml for tibolone. The calibration curves were constructed using a simple and linear weighted (1/concentration^2) least square regression to achieve homogeneity of variance. The correlation coefficients were ≥0.9990 (*n*=6) for tibolone. The linearity plot of the calibration curve is presented in figure-6.

3.6. Sensitivity (lower limit of quantification)

The LOQ is defined as the lowest concentration of the calibration standard yielding accuracy ±20% and precision of ≤20%. The LOQ for tibolone was 10.178pg/ml. The intra-run precision (% RSD) at the LOQ plasma samples containing tibolone was 0.37%. The intra-run accuracy (% RE) at the LOQ plasma samples containing tibolone was –0.07%.

3.7. Precision and Accuracy

The intra-run precision (*n*=6) ranged from 2.04 to 3.18% at HQC, MQC & LQC levels and 7.04% at LLOQQC level, intra-run accuracy(%RE) at HQC, MQC & LQC levels ranged from –4.04 to 2.07% and –0.61% at LLOQQC level for Tibolone. The inter-run precision and accuracy were determined by pooling all individual assay results of replicate (*n*=24) QC samples over the four separate analytical runs. The inter-run precision (% RSD) ranged from 4.01 to 5.69% and at LLOQQC level it was 5.10%. The inter-run accuracy (% RE) ranged from –5.31 to –0.84% and at LLOQQC level it was –0.10% for Tibolone. The results of intra-run and inter-run precision and accuracy are tabulated in Table-3a & 3b respectively.

3.8. Recovery

Six replicates at low, medium and high-quality control concentrations for tibolone were prepared for recovery determination. The mean recovery for tibolone and Δ-tibolone D6 were 72.88% and 99.28% respectively.

3.9. Stability

The results of the stability studies are enumerated in **Table 4**. The bench top stability results allowed us to conclude that tibolone is stable for 7 hours at room temperature. The in-injector stability at 10°C was established for 62 hours. The freeze-thaw stability results concluded that on even after five complete cycles of freezing and thawing there is no impact on the stability of tibolone when stored at –75°C. The interim Long-term stability of tibolone in plasma in storage at–75°C is established for about 24 days (Table-4).

3.10. Matrix effect

The matrix factor for tibolone and Δ-tibolone D6 was calculated by comparing the area response observed in post spiked samples with that of unextracted derivatized samples at LQC, MQC and HQC level and the matrix effect was evaluated from the % RSD of matrix factor at each level. Three quality control samples at each level were analyzed and the mean of % bias of the samples analyzed was found less than 15% for each QC level for tibolone and Δ-tiboloneD6 and presented in Table 5.

Hence, this clearly proved that the elution of endogenous matrix peaks during the run has no effect on the quantification of tibolone. Therefore, the method of extraction of tibolone from plasma was rugged enough and gave accurate and consistent results when applied to subject samples.

3.11. Dilution Integrity

The dilution integrity experiment was intended to validate the dilution test to be carried out because of high analyte concentrations (above ULOQ), which may be observed during real subject samples analysis. Dilution integrity experiment was performed at about 1.60 times the ULOQ concentration. Six replicates samples of ½ and ¼ dilution concentrations were prepared and the back-calculated concentrations were derived by applying the dilution factor of 2 and 4 respectively against the freshly prepared calibration curve.

3.12. Application of Method

The analytical method validated was applied to determine the concentrations of tibolone. A clinical study was conducted to evaluate the bioequivalence of two formulations of Tibolone for an oral contraceptive formulation. The study design was a crossover of three different formulations in three periods to evaluate the relative bioavailability of tibolone. All volunteers have given informed consent for participation, wherein the volunteers were housed and plasma samples were periodically collected up to 24 hours after a single oral dose administration of 2.50mg tablet of tibolone to 18 healthy human adult postmenopausal female volunteers in each phase. Blood sample were collected each period were collected at 0.00 (pre-dose), 0.33, 0.50, 0.67, 0.87, 1.00, 1.25, 1.50, 1.75, 2.00, 2.50, 3.00, 4.00, 5.00, 6.00, 8.00, 10.00, 12.00 and 24.00h after oral dose administration of a 2.50mg tablet. A volume of 7 mL blood was collected each time in vacutainer containing K_2EDTA. A total of

19-time points were collected in each period and plasma was separated by centrifugation at 3800 rpm, 10°C for ten minutes and stored below -75 °C until sample analysis.

All subjects were analyzed with time points of both the periods processed together. 1026 human plasma samples were analyzed in 14 runs including repeat sample reanalysis. All in-study result data calibration standards and quality control samples were within the acceptance criteria. The obtained results were also in accordance with the literature data confirming the method. The mean tibolone plasma concentration–area ratio following the 2.50mg oral dose of tibolone to human subjects is shown in Figure-7. Twenty calibration curves were processed for quantification of analyzed samples including repeat analysis of samples due to analytical anomalies. No interference peak was found in pre-dose samples for all volunteers and the assay was found to be accurate and reproducible.

The mean (±SD) plasma maximum concentrations obtained were 1612.71 (±848.45), 1792.54(±998.80) and 1406.21 (±874.10) pg/ml, for the tibolone for Reference 1, Reference 2 and Test formulations respectively. Graphical representations of mean tibolone plasma concentration–time profile following a 2.50mg tablet oral dose of tibolone in postmenopausal healthy female human subjects are represented in Figure–8.

4.0 CONCLUSIONS

This article reports a novel and robust method for determination of tibolone by oxime derivatization and with the use of Δ-tibolone D6 as internal standard the selectivity of the method for the isobaric metabolite Δ-tibolone is also established. The signal–to–noise of greater than 10 assures enables the method can be even detected to even lower concentrations for future requirements of low dose formulations. The SPE–Derivatization extraction is the critical step in the sample preparation assures a better sample cleanup for the determination of tibolone in human plasma with a limit of quantification of 10.178pg/ml for tibolone.

It is concluded that this method is sensitive, specific and reproducible for the quantitative determination of tibolone in human plasma in pharmacokinetic and bioavailability studies.

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Table I	**Ion source and analyte-dependent parameters**	
Ion source		
Capillary voltage	2.0 kV	
Source temperature	150ºC	
Desolvation Temperature	500ºC	
Desolvation gas flow	1000 L/Hr	
Polarity mode	Positive	
Analyte dependent parameters		
	Derivatized Tibolone	Derivatized Δ-Tibolone D6
Precursor ion (m/z)	328.2	334.3
Product ion (m/z)	295.2	129.2
Cone voltage (V)	40	40
Q1-HM / LM* (amu)[a]	15.0 / 2.8	15.0 / 2.8
Q3-HM / LM* (amu)[b]	14.9 / 3.0	14.9 / 3.0
Collision energy (V)	14	32
[a] Quadrupole 1 High and Low Mass resolution parameters		
[b] Quadrupole 3 High and Low Mass resolution parameters		

Table II: Selectivity in the presence of Metabolites

Sr. No.	Sample Type	Analyte Response at RT of Tibolone	% Interference	Response at the RT of Δ-Tibolone D6)	% Interference
1	BLANK	0	0.00	0	0.00
2	BLANK	0	0.00	0	0.00
3	BLANK (with OTC)	0	0.00	0	0.00
4	BLANK (with OTC)	0	0.00	0	0.00
5	BLANK (with M1+M2)	0	0.00	0	0.00
6	BLANK (with M1+M2)	0	0.00	0	0.00
7	BLANK (with M3 only)	0	0.00	0	0.00
8	BLANK (with M3 only)	0	0.00	0	0.00
9	BLANK (with Δ-TIB D6	0	0.00	138690	-
10	BLANK (with Δ-TIB D6	0	0.00	141465	-
11	ULOQ (with TIB only)	235446	-	0	0.00
12	ULOQ (with TIB only)	238452	-	0	0.00
13	ULOQ (with TIB+M1+M2+M3)	240562	-	0	0.00
14	ULOQ (with TIB+M1+M2+M3)	231168	-	0	0.00

M1= 3α Hydroxy Tibolone; M2=3ß Hydroxy Tibolone; M3=Δ-Tibolone:: OTC= Paracetamol, Ranitidine, Ibuprofen, Aspirin

Table IIIa **Intra-run or within batch (*n*=6) precision and accuracy of Tibolone in human plasma**

Analyte	QC level	Spiked conc. (pg/mL)	Mean (±SD) calculated conc. (pg/mL)	% CV	% RE
Tibolone	LLOQQC	10.342	10.279 (±0.724)	7.04	-0.61
	LQC	27.953	26.823 (±0.853)	3.18	-4.04
	MQC	901.699	886.996 (± 26.665)	3.01	-1.63
	HQC	1595.927	1629.014 (±33.171)	2.04	2.07

Table IIIb **Inter-run or between batch(*n*=24) precision and accuracy of Tibolone in human plasma**

Analyte	QC level	Spiked conc. (pg/mL)	Mean (±SD) calculated conc. (pg/mL)	% CV	% RE
Tibolone	LLOQQC	10.342	10.331 (±0.527)	5.10	-0.10
	LQC	27.953	26.468 (±1.273)	4.81	-5.31
	MQC	901.699	881.659 (±35.329)	4.01	-2.22
	HQC	1595.927	1582.521 (±89.982)	5.69	-0.84

Table IV	**Stability results for Tibolone (*n*=6 for each stability experiment)**					
Stability	Spiked conc. (pg/mL)	Stability samples (pg/mL)		Comparison samples (pg/mL)		Change (%, Stability)
		Mean	± SD	Mean	± SD	
Bench top [a]	1595.927	1629.014	33.171	1542.729	94.513	105.59
	901.699	862.470	42.979	841.680	59.652	102.47
	27.953	25.679	1.178	26.906	0.710	95.44
Process stability [b]	1595.927	1575.822	113.789	1629.031	33.127	96.73
	901.699	886.977	26.689	895.493	31.318	99.05
	27.953	26.971	0.960	26.903	0.706	100.26
Freeze/thaw [c]	1595.927	1499.407	108.346	1595.680	43.656	93.97
	901.699	886.996	26.665	862.490	42.978	102.84
	27.953	27.696	1.251	26.823	0.853	103.25
Long-term Stability [d]	1595.927	1563.716	93.196	1629.014	33.171	95.99
	901.699	862.49	42.978	889.184	31.318	96.31
	27.953	28.724	1.023	27.699	1.225	103.70
** - %Stability – Determined by calculating the percentage of mean stability QC concentration against mean comparison QC concentrations*.						
[a]n After 7h at room temperature.						
[b] After 62h in autosampler at 10ºC.						
[c] After five freeze/thaw cycles at –30ºC.						
[d] Long-term matrix stability at –30ºC for 24 days.						

Table V Matrix effect (*n*=8) for tibolone and Δ-tibolone D6

		HQC			MQC			LQC		
Sr. No.	Parameters	Mean matrix factor	(± SD)	% CV	Mean matrix factor	(± SD)	% CV	Mean matrix factor	(± SD)	% CV
1	Analyte area	0.942	0.048	1.41	1.024	0.99	1.05	1.109	0.064	2.05
2	IS area	0.961	0.046	1.36	1.078	0.065	1.28	1.058	0.044	1.22
3	IS normalized	0.981	0.012	0.82	1.126	0.048	0.95	1.097	0.049	1.02

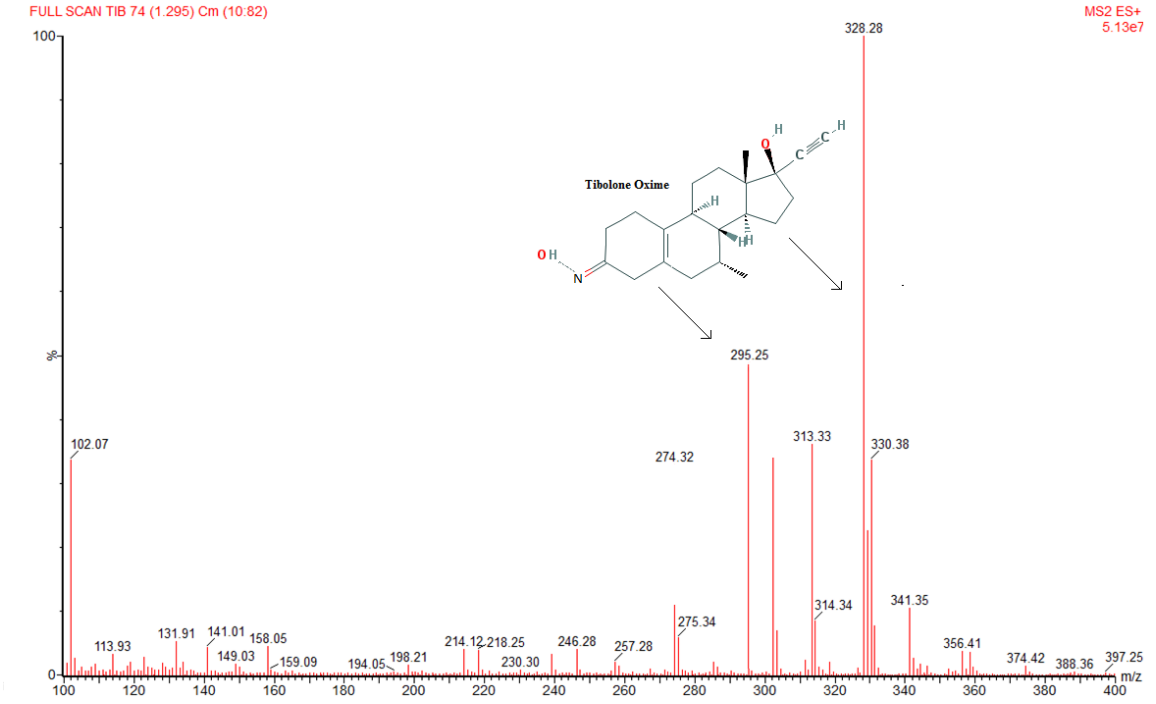

Figure 1: Product ion spectra deravatised precursor ion of tibolone

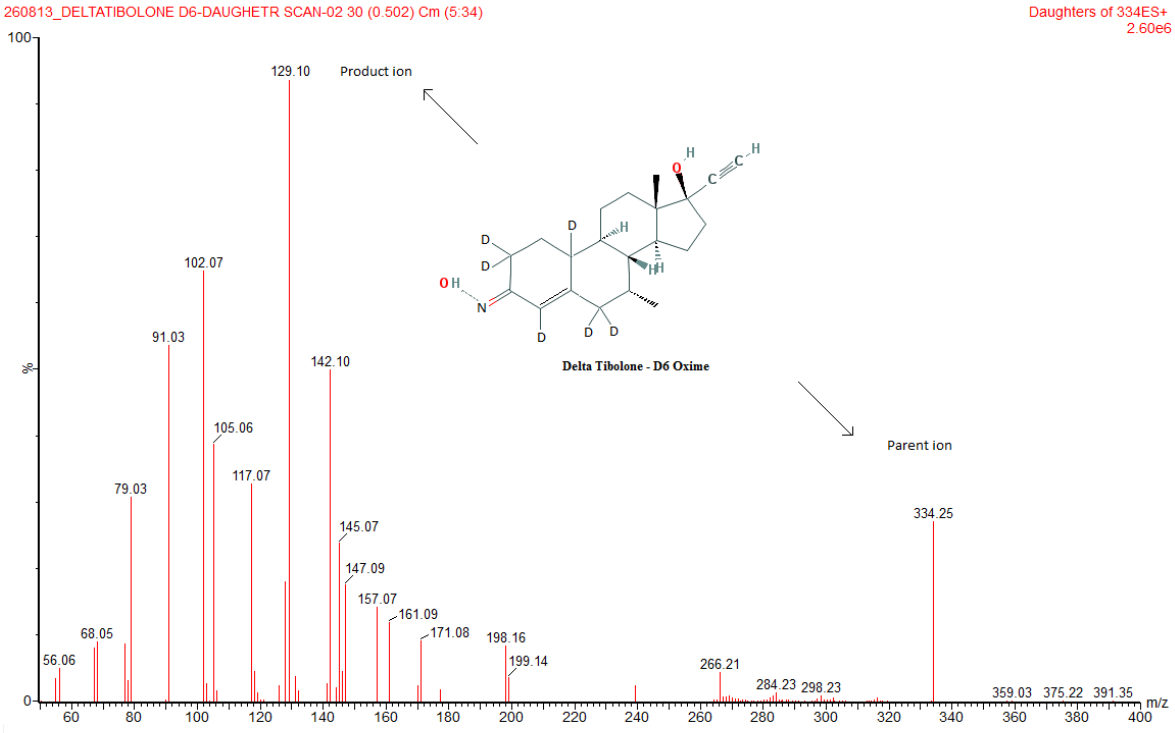

Figure 2: Product ion spectra of deravatised precursor ion of delta tibolone D6

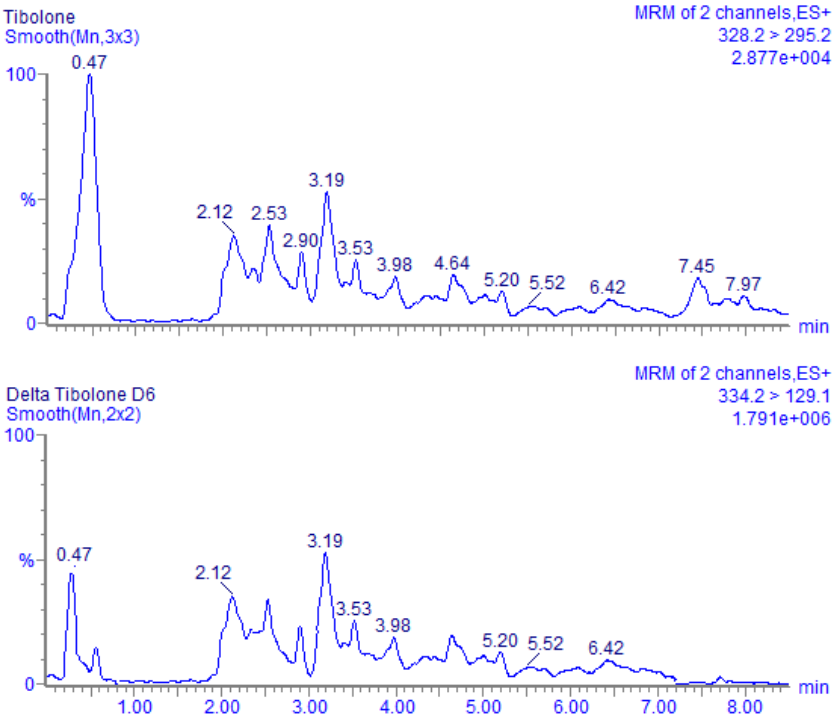

Figure 3: Representative Chromatogram of an Extracted blank sample

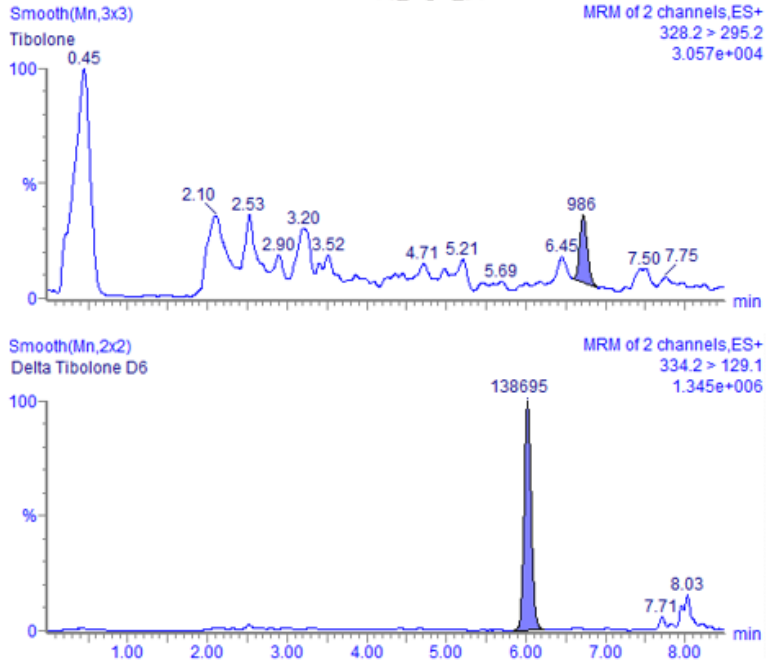

Figure 4: Representative chromatogram of extracted LLOQ sample

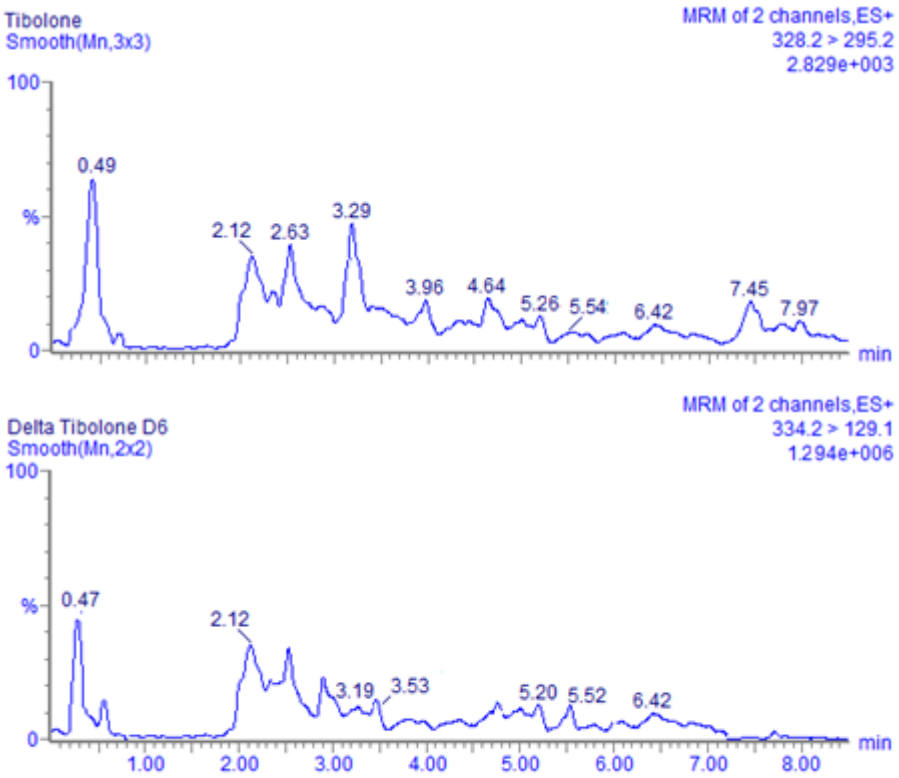

Figure 5 Representative Chromatogram of blank sample in the presence of its Hydroxy metabolites

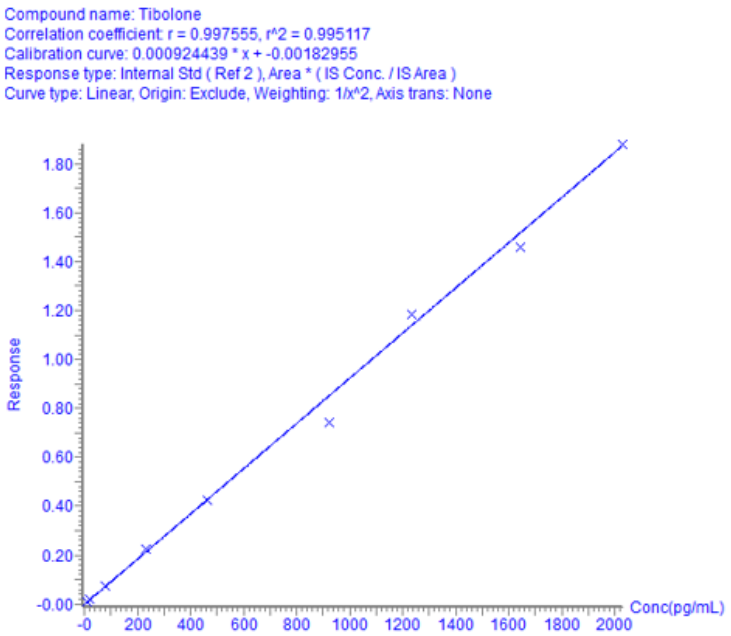

Figure-6: Area ratio Vs Concentration curve plot of Tibolone

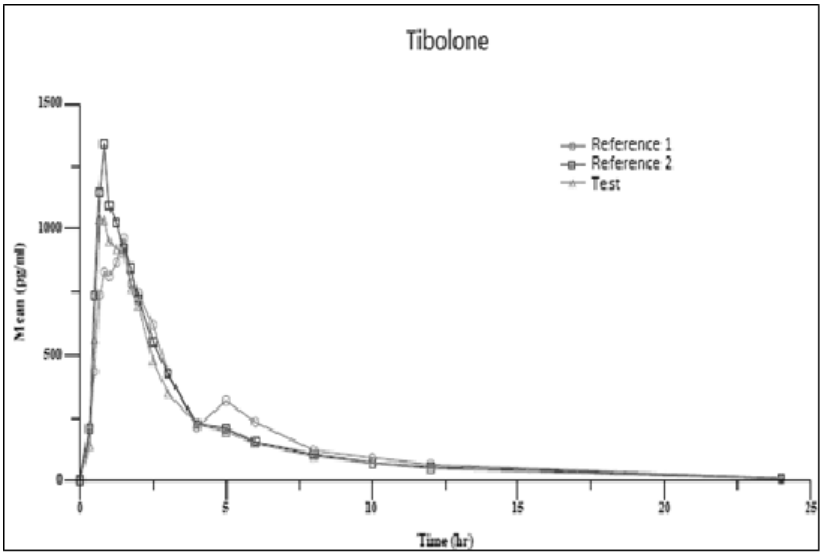

Figure-7: Mean plasma concentration Vs Time plot for Tibolone

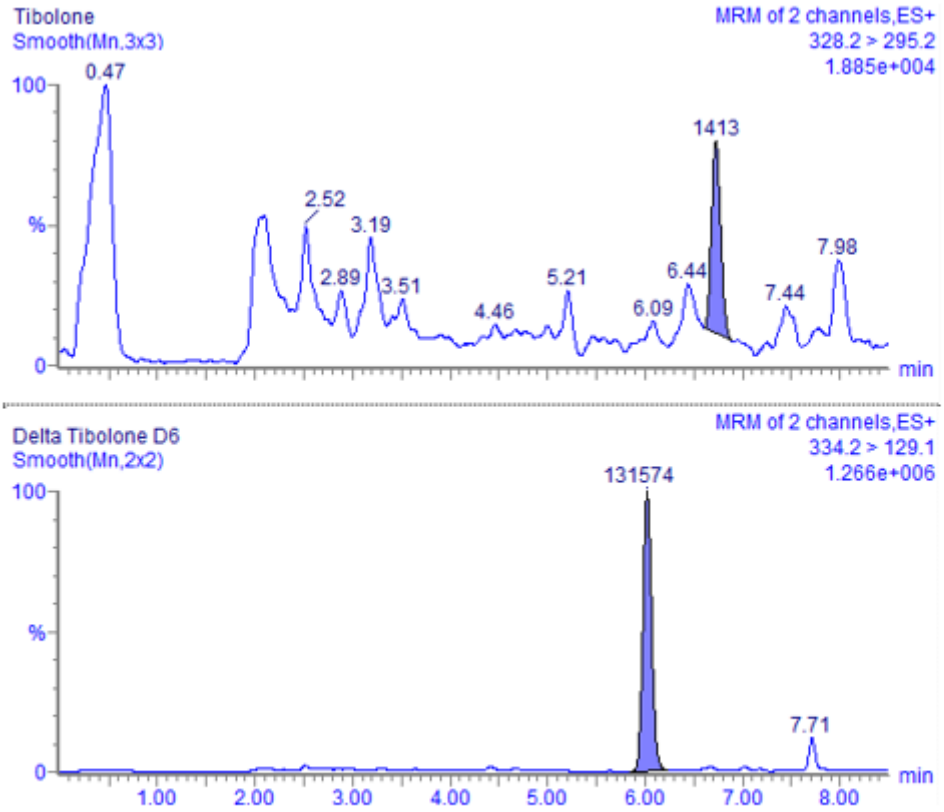

Figure-8: Representative chromatogram of Tibolone in subject sample