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Quantification of 3α-hydroxytibolone in human plasma by high performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS): Application in a bioequivalence study in healthy postmenopausal volunteers

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ABSTRACT

A sensitive, specific and fast method to quantify 3α-hydroxytibolone in human plasma using deuterated 3α-hydroxytibolone (d5) as internal standard is described. The analyte and the internal standard were extracted from plasma (900 μL) by liquid-liquid extraction using ethyl ether/hexane (50/50, v/v) and ammonium hydroxide (50%). The extracts were analyzed by high performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry without derivatization. Chromatography was performed isocratically on a Gemini-NX™ C_{18} 5 μm (150 × 4.6 mm i. d.) column. The method had a chromatographic run time of 3.75 min and a linear calibration curve over the range 1–100 ng/mL. The limit of quantification validated was 1 ng/mL. This method was used to assess the bioequivalence between two different tibolone oral formulations: Livolon (1.25 mg tablet) provided by Biolab Sanus Farmacêutica (Brazil), as the test formulation, and Libiam™ (1.25 mg tablet) produced by Libbs Farmacêutica (Brazil), as the reference formulation. A single 3.75 mg dose of each formulation was administered to 46 postmenopausal female healthy volunteers. The study was conducted in an open, randomized, two-period crossover balanced design with a 2 week washout interval between the doses. The 90% confidence interval for C_{max}, $AUC_{(0\text{-last})}$ and $AUC_{(0\text{-inf})}$ individual test/reference ratios were 97.48–111.51, 95.35–103.20 and 96.42–103.86, respectively. It is concluded that Livolon (1.25 mg tablet) is bioequivalent to Libiam™ (1.25 mg tablet), with regards to both rate and extent of absorption.

1. Introduction

Tibolone [(7α,17α)-17-hydroxy-7-methyl-19-norpregn-5 (10)-en-20-yn-3-one] is a synthetic steroid with estrogenic, progestogenic and androgenic effects. It has been widely used as an alternative to estrogen replacement therapy for almost two decades, mainly to treat postmenopausal symptoms (e.g., hot flushes, insomnia, headache, fatigue, mood swings and decreased libido) and prevent osteoporosis. A daily dose of 2.5 mg is often recommended but lower doses such as 1.25 mg are also used [1,2].

After oral administration to postmenopausal women, tibolone is rapidly converted into three active metabolites: 3α-hydroxytibolone (predominant metabolite), 3β-hydroxytibolone and Δ^4-tibolone. The metabolism occurs in liver and intestine as well as in tibolone target tissues. The 3-hydroxy metabolites are present in the blood circulation predominantly in their inactive form (sulphate conjugates). The plasma levels of tibolone and the Δ^4-isomer are very low [3]. Bioequivalence studies are based on the quantification of 3α-hydroxytibolone in plasma, as recommended by regulatory agencies, since it is the predominant active metabolite of tibolone [4,5].

The objective of this study was to develop a sensitive, specific and fast high performance liquid chromatography (HPLC) coupled

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to electrospray ionization (ESI) tandem mass spectrometry (MS/MS) method to quantify 3α-hydroxytibolone in human plasma using deuterated 3α-hydroxytibolone (d5) as internal standard (I.S.), without derivatization. The method was used in a bioequivalence assay between two different tibolone oral formulations: Livolon (1.25 mg tablet) provided by Biolab Sanus Farmacêutica (Brazil), as the test formulation, and Libiam™ (1.25 mg tablet) produced by Libbs Farmacêutica (Brazil), as the reference formulation.

2. Materials and methods

2.1. Method development

2.1.1. Chemicals, reagents and blank human blood

Livolon, the tibolone test formulation, was provided by Biolab Sanus Farmacêutica (Brazil), with lot number 4030497. Libiam™, the tibolone reference formulation, was purchased from Libbs Farmacêutica (Brazil), with lot number 13k0524. 3α-hydroxytibolone was purchased from Toronto Research Chemicals (Canada), with lot number 25-SSR-16-1 and purity of 97%. 3-hydroxytibolone-d5 (a mixture of deuterated 3α and 3β isomers) was purchased from Synfine Research (Canada), with lot number S-1315-001A4 and purity of 98.6%. Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Panreac (Spain). Sodium hydroxide (Panreac, Spain), ammonium hydroxide (Synth, Brazil), ethyl ether (Honeyhell, U.S.A.) and hexane (Honeyhell, U.S.A) were of analytical grade. Ultra pure water was obtained from a MilliQ™ system (EMD Millipore, U.S.A.).

Blank human blood was collected with the anticoagulant sodium heparin from healthy and drug-free volunteers. After centrifugation, blank human plasma was obtained, pooled and stored at −20 °C until used.

2.1.2. Calibration standard solutions and quality control samples

Stock solutions of 3α-hydroxytibolone and 3-hydroxytibolone-d5 were prepared in methanol/water (70/30, v/v). Working solutions were prepared by diluting stock solutions with acetonitrile/water (50/50, v/v).

Calibration standard solutions were prepared by spiking blank plasma with 3α-hydroxytibolone working solutions to give concentrations of 1, 2, 10, 20, 40, 60, 80 and 100 ng/mL. The calibration standard solutions and blanks were daily prepared in duplicate for each analytical batch.

Quality control (QC) samples were prepared by spiking blank plasma with 3α-hydroxytibolone working solutions to give concentrations of 1, 3, 5, 30, 75 and 150 ng/mL, designated LOQ (limit of quantification), QCL (low concentration quality control), QCM 1 (medium concentration quality control 1), QCM 2 (medium concentration quality control 2), QCH (high concentration quality control) and QCD (dilution quality control), respectively. The QCD (used to evaluate the dilution integrity of the method) was diluted in blank plasma (1:1) for analysis. QC samples were daily prepared for each analytical batch.

The spiked plasma samples (calibration standards and quality controls) were extracted from each analytical batch along with the unknown samples.

2.1.3. Sample preparation

Briefly, 900 μL of each plasma sample were introduced into a glass tube with 50 mL of the 3-hydroxytibolone-d5 working solution (1000 ng/mL), 50 mL of ammonium hydroxide (50%) and 4 mL of ethyl ether/hexane (50/50, v/v), sequentially and with 5 s of vortex mixing in between. Then, the glass tube was vortex-mixed for 60 s and frozen at − 80 °C. The upper phase (organic phase) was transferred to another glass tube and dried with N_2 at 40 °C. The dry residues were reconstituted with 120 mL of acetonitrile/water (60/40, v/v) + NaOH (2 mM) and vortex-mixed for 10 s. At this point, the solutions were transferred to the CTC HST PAL™ auto-injector microvials (CTC Analytics, Switzerland).

2.1.4. Chromatographic conditions

The HPLC was performed in a Gemini-NX™ C_{18} 5 μm (150 × 4.6 mm i. d.) column (Phenomenex, U.S.A.) with a G1311A pump (Agilent, Germany) at a flow rate of 1150 mL/min. The mobile phase (isocratic elution) was acetonitrile/water (60/40, v/v) + NaOH (2 mM). The column was operated at 60 °C and the pressure of the system was approximately 67 bar. The temperature of the auto sampler was maintained at 8 °C and the injection volume was 20 μL. Typical retention time was approximately 2.5 min for both 3α-hydroxytibolone (analyte) and 3α-hydroxytibolone-d5 (I.S.) and the total run time was 3.75 min. The total flow rate was split in a 2:3 ratio (no more than 770 mL/min were directed to the ESI source). The smooth factor of 5 was set for both analyte and I.S. All chromatograms were integrated in the automatic mode by Analyst™ software version 1.4.1 (Applied Biosystems, U.S.A.).

2.1.5. Mass-spectrometric conditions

The MS was performed in a API 4000™ mass spectrometer (Applied Biosystems, U.S.A.) equipped with an ESI source using a crossflow counter electrode run in positive ion mode with multiple reaction monitoring (MRM). The mass spectrometer was set as follows: 332.2 > 297.2 and 337.4 > 302.4 for 3α-hydroxytibolone and I.S. as the precursor ions and the respective product ions (*m/z*). The proposed fragmentation pathways are presented in Figs. 1 and 2. Full-scan mass spectrograms of 3α-hydroxytibolone and I.S. are illustrated in Figs. 3 and 4, respectively. The source temperature was set at 600 °C and the ionspray voltage at 5.0 kV. For both analyte and I.S., the dwell time and the optimized parameters of declustering potential, collision energy and collision exit potential were 0.4 s, 46 V, 12 eV and 18 V, respectively. Data were acquired by Analyst™ software version 1.4.1 (Applied Biosystems, U.S.A.).

2.2. Method validation

Samples analyses were carried out in accordance with the bioanalytical methods validation guidelines from the U.S. Food and Drug Administration (F.D.A.) [4] and Brazilian National Health Surveillance Agency (A.N.V.I.S.A.) [5].

2.2.1. Linearity

A linear least-squares regression with a weighting index of 1/x was performed on the peak area ratios of 3α-hydroxytibolone and I.S. vs. 3α-hydroxytibolone concentrations of the eight plasma calibration standards (1, 2, 10, 20, 40, 60, 80 and 100 ng/mL) in duplicate to generate the calibration curves. In addition, a blank plasma sample (non-spiked) and a zero plasma sample (spiked only with I.S.) were run in duplicate to demonstrate the absence of interferences. Correlation coefficient must be greater than 0.98.

2.2.2. Accuracy and precision

QC samples were used to evaluate the accuracy and precision of the method. Accuracy was defined as the percentage relative error, RE (%) = (E − T) (100/T), and precision as the relative standard deviation, RSD (%) = 100 (SD/M), where M is the mean, SD is the standard deviation of M, E is the experimentally determined concentration and T is the theoretical concentration. Accuracy must be between 85 and 115% of the nominal concentration and precision must be less than 15% (except for LOQ, as described below; item 2.2.3).

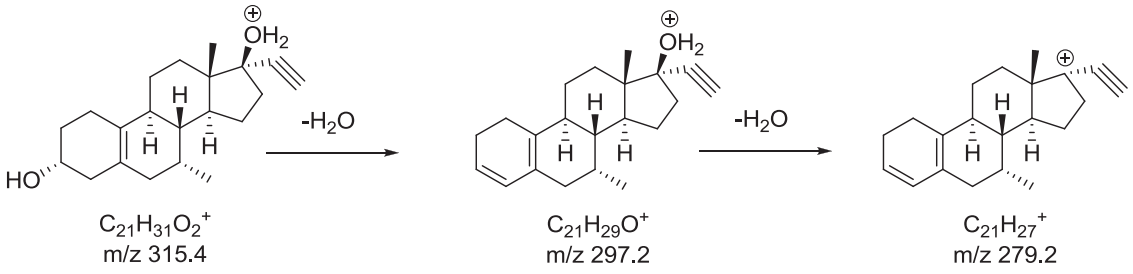

Fig. 1. Proposed pathway for the fragmentation of 3α-hydroxytibolone (analyte).

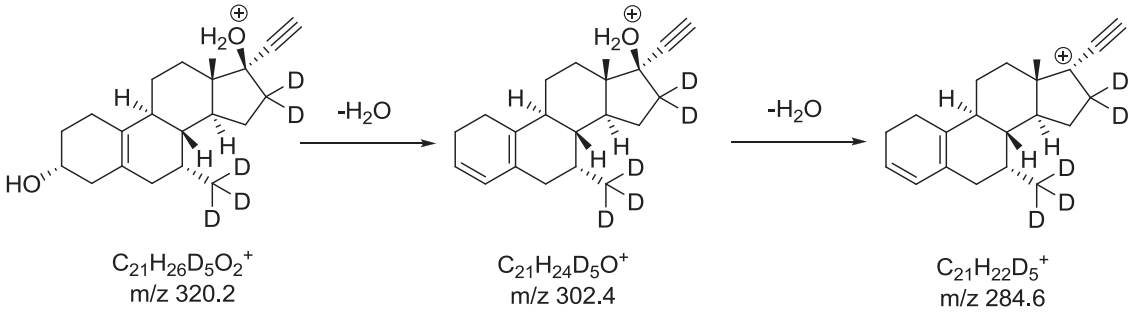

Fig. 2. Proposed pathway for the fragmentation of 3α-hydroxytibolone-d5 (internal standard).

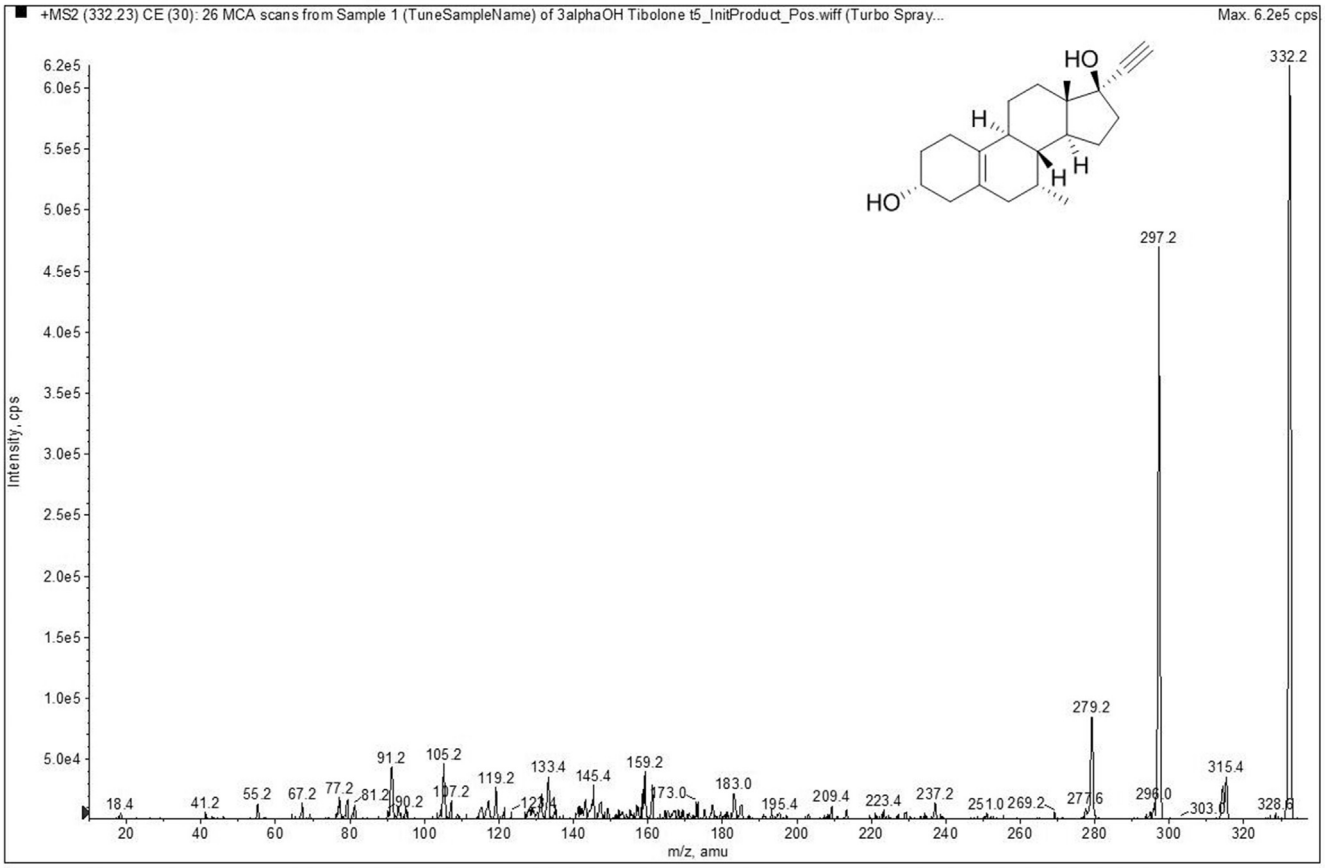

Fig. 3. Full scan mass spectra of 3α-hydroxytibolone (analyte).

2.2.3. Selectivity and sensitivity

Blank normal plasma (n = 4), blank lipemic plasma (n = 2) and blank hemolyzed plasma (n = 2) samples from different donors were run during the pre-study validation to evaluate selectivity. No

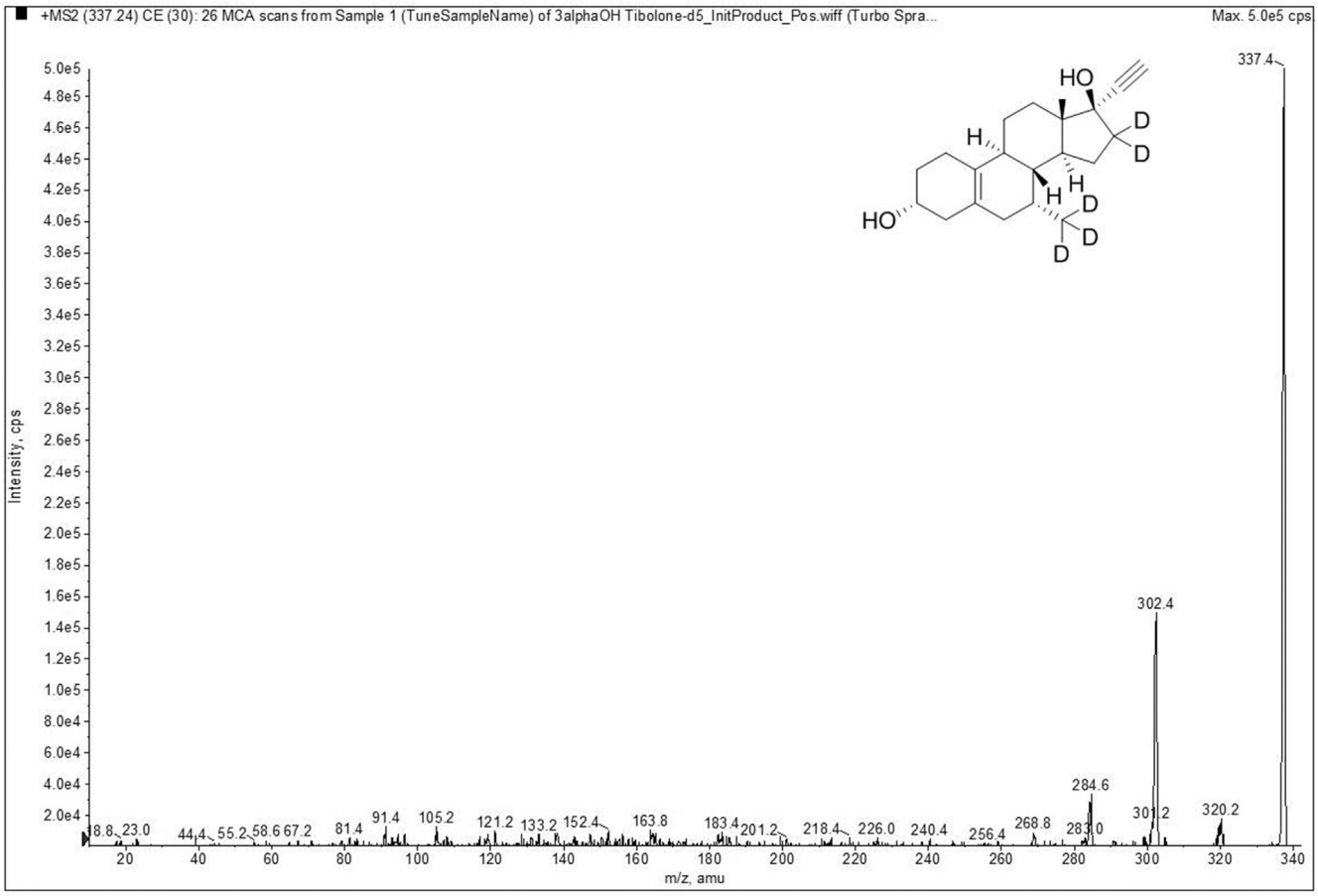

Fig. 4. Full scan mass spectra of 3α-hydroxytibolone-d5 (internal standard).

peaks at the retention times of the analyte and I.S were expected. The LOQ validated was the lowest concentration at which both the accuracy and precision were less than 20% and the analyte response was at least 5 times the baseline noise.

2.2.4. Carry-over and matrix effect

Blank normal plasma samples (n = 2) were run after an injection of a QCH sample to evaluate carry-over. Carry over must be less than 20% and 5% of the LOQ of the analyte and I.S., respectively. QCL and QCH samples made from normal plasma (n = 12 for each concentration), lipemic plasma (n = 6 for each concentration) and hemolyzed plasma (n = 6 for each concentration) were run to evaluate matrix effect. Then, a factor matrix standard (FMS) was obtained for each sample according to the following formula: FMS = (response of analyte in matrix/response of internal standard in matrix)/(response of analyte in solution/response of internal standard in solution). The precision of FMS must be less than 15%.

2.2.5. Stability

QCL (n = 3) and QCH (n = 3) samples were subjected to three freeze-and-thaw cycles (from −20 °C to room temperature), short-term (6:15 h at room temperature), long-term (thawed and extracted to be tested after 186 days) and autosampler (63:30 h at 8 °C) stability tests. Subsequently, the analyte concentrations were measured in comparison with freshly prepared samples (n = 3 for each concentration) to obtain accuracy and precision.

2.3. Bioequivalence study

2.3.1. Clinical protocol

The present method was used in a bioequivalence assay between two different tibolone oral formulations: Livolon (1.25 mg tablet) provided by Biolab Sanus Farmacêutica (Brazil), as the test formulation, and Libiam™ (1.25 mg tablet) produced by Libbs Farmacêutica (Brazil), as the reference formulation. Forty-eight postmenopausal female volunteers aged between 46 and 60 years old and within 15% of the ideal body weight were selected for the study. All volunteers were drug-free and healthy as assessed by general physical examination, electrocardiogram and laboratory tests (biochemical, hematological, serological and routine urinalysis). All subjects provided written informed consent and the study was conducted in accordance to the provisions of the Declaration of Helsinki (1964) revisions [6].

The study was conducted in an open, randomized, two-period crossover balanced design with a 2 week washout interval between the doses. During each period (approximately 36 h), the volunteers were hospitalized at 6:00 p.m. having a standard evening meal between 7:00 and 9:00 p.m. After an overnight fast they received at 7:00 a.m. a single 3.75 mg dose (3 tablets of 1.25 mg) of either formulation with 200 mL of water. All volunteers were then fasted for 2 h following the drug administration after which a standard breakfast was consumed. A standard lunch and an evening meal were provided throughout the day. No other foods were permitted during the in-house period. Signs, symptoms, arterial pressure, heart rate and temperature were recorded just before and every 4 h after drug administration. Blood samples (8 mL) from a

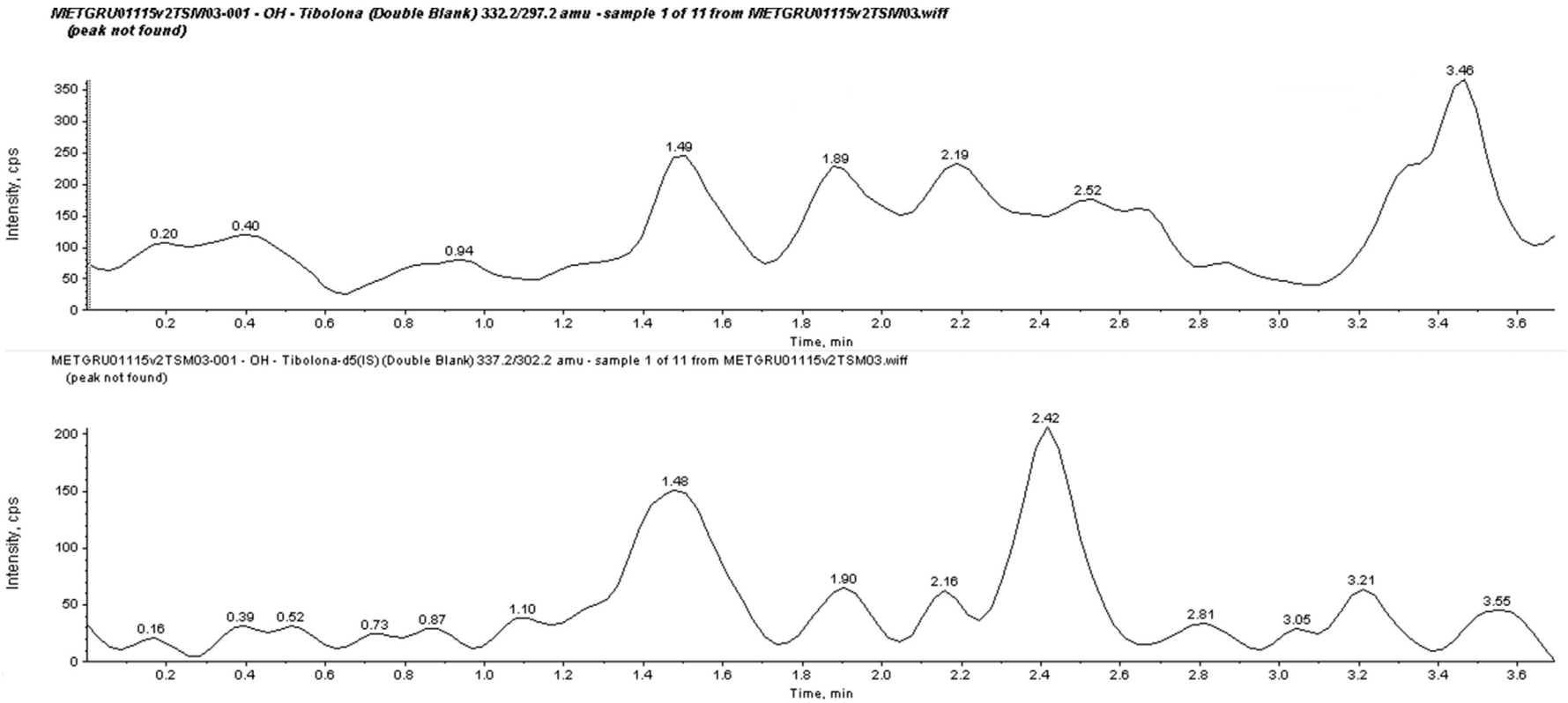

Fig. 5. Chromatogram at blank normal plasma (top channel: 3α-hydroxytibolone/bottom channel: internal standard).

Table 1
Quality control samples accuracy and precision data from the pre-study validation (n = 7 for each concentration/3 batches).* diluted in blank plasma (1:1) for analysis.

	Nominal concentration (ng/mL)					
	1	3	5	30	75	150*
Intra-batch (n = 7)						
Mean	1.04	3.30	5.43	31.90	79.40	79.20
Range	0.80–1.24	3.02–3.67	4.85–5.82	31.30–32.60	77.30–83.00	76.50–82.80
Accuracy (%)	104.40	110.00	108.50	106.20	105.90	105.60
Precision (%)	13.20	7.10	7.50	1.60	2.60	3.00
Inter-batch (n = 21)						
Mean	0.97	3.07	5.07	30.50	76.30	75.10
Range	0.70–1.26	2.72–3.67	4.51–5.82	27.30–32.60	69.10–83.00	69.00–82.80
Accuracy (%)	97.90	102.40	101.30	101.80	101.70	100.10
Precision (%)	14.70	7.70	8.20	5.50	4.90	5.20

suitable antecubital vein were collected in sodium heparin containing tubes before and after 0.25, 0.5, 0.75, 1, 1.25, 1,5, 1.75, 2, 2.33, 2.66, 3, 3,33, 3.67, 4, 4.5, 5, 5.5, 6, 8, 10, 12, 14, 16, 18 and 24 h after the administration of each tibolone formulation. After centrifugation of the blood, plasma was obtained and stored at −20 °C until used.

2.3.2. Pharmacokinetics and statistical analysis

The bioequivalence between the two formulations of tibolone was assessed by calculating individual test/reference ratios for the peak plasma concentration (C_{max}), the area under the curve (AUC) of the plasma concentration until the last concentration observed ($AUC_{(0\text{-last})}$) and the area under the curve of the plasma concentration between the first sample and infinity ($AUC_{(0\text{-inf})}$). The C_{max} and the time taken to achieve this concentration (T_{max}) were obtained directly from the curves. The $AUC_{(0\text{-last})}$ was calculated by applying the linear trapezoid rule. Extrapolation of this area to infinity ($AUC_{(0\text{-inf})}$) was done by adding the value C_{last}/k_e to the calculated $AUC_{(0\text{-last})}$. The first-order terminal elimination rate constant (k_e) was estimated by linear regression from the points describing the elimination phase in a log-linear plot, and the half-life ($t_{1/2}$) was derived from this rate constant ($t_{1/2} = \ln(2)/k_e$).

The C_{max} and AUC data for the two tibolone formulations were analyzed by ANOVA to determine whether the 90% CI (confidence interval) of the individual test/reference ratios was within the 80–125% interval indicative of bioequivalence.

3. Results

3.1. Method validation

Blank normal plasma, blank lipemic plasma and blank hemolyzed plasma samples were run during the pre-study validation and there were no interferences at the retention times of the analyte or I.S. Fig. 5 shows a chromatogram at blank normal plasma. The method was a linear regression for 3α-hydroxytibolone concentrations from 1 to 100 ng/mL (correlation coefficient ranged from 0.9991 to 0.9994). QC samples intra and inter-batch accuracy and precision from the pre-study validation are summarized in Table 1. The LOQ validated was 1 ng/mL.

Fig. 6 shows a chromatogram at the LOQ. Although a mixture of 3α and 3β-hydroxytibolone-d5 was employed as internal standard, these metabolites have different chemical and physical properties since they are diastereomers [7]. In RP chromatography, 3α-hydroxytibolone-d5 is more polar [8], eluting earlier (retention time at 2:51 min) than 3β-hydroxytibolone-d5 (retention time at 2.86 min). Indeed, in the analyte channel the first peak was present in most of the samples whereas the later peak appeared only near the T_{max}, indicating that the first peak is related to 3α-hydroxytibolone, which has been reported as the predominant circulating metabolite of tibolone [3,7,8].

Carry-over evaluation shown no peaks of analyte or I.S in the chromatograms of blank normal plasma samples run after an injection of a QCH sample (Fig. 7). No significant matrix effect was observed (the CV of FMS was 4% - data not shown). The stability tests indicated no significant degradation of QC samples (accuracy between 85 and 115% of the nominal concentration and precision less than 15% - data not shown).

3.2. Bioequivalence study

Two volunteers dropped-out, both for personal reasons, one before the beginning of the study and other during the second in house period. Therefore, forty-six volunteers completed the clinical study. Tolerance of both tibolone formulations was good. General physical examination, electrocardiogram and laboratory tests were repeated at the end of the study and the results showed no relevant alterations. The pharmacokinetic parameters found after a single oral dose (3.75 mg) of each tibolone formulation (shown in Table 2) were similar to those reported in the literature for postmenopausal women. The mean 3α-hydroxytibolone plasma concentrations versus time profiles of each tibolone formulation are shown in Fig. 8. Table 3 summarizes the statistical analysis of C_{max}, $AUC_{(0\text{-last})}$ and $AUC_{(0\text{-inf})}$ individual test/reference ratios.

4. Discussion

Pioneering studies on the in vivo human metabolism of tibolone were based on liquid chromatography (LC) coupled to radioactivity detection [9]. Later, a series of studies of the pharmacokinetics of tibolone used a gas chromatography (GC) coupled to chemical ionization MS (GC-CI-MS) method with 3α-hydroxytibolone-d5 as I.S (LOQ: 0.1 ng/mL; retention times were not reported) [10–15]. Another GC method, coupled to electron impact high resolution MS (GC-EI-HRMS), was developed. Ethisterone was employed as I.S (LOQ: 0.02 ng/mL; retention time: approximately 11 min) [16]. At last, a GC-MS method with 1,2-testosterone-d5 as I.S was developed (LOQ: 0.5 ng/mL; retention times were not reported) [17]. The majority of the GC methods described above employed solid-phase extraction and all of them used derivatization to increase the volatility of the compounds of interest.

LC-MS and LC-MS/MS have become the most common techniques employed for the quantification of steroids in biological matrices as an alternative to GC-MS, which has important limitations. GC-MS, in which some compounds thermally decompose and derivatization is often needed, has longer run times and it is not suitable for conjugated steroids [18,19].

Table 4 summarizes the analytical details of the LC-MS and LC-

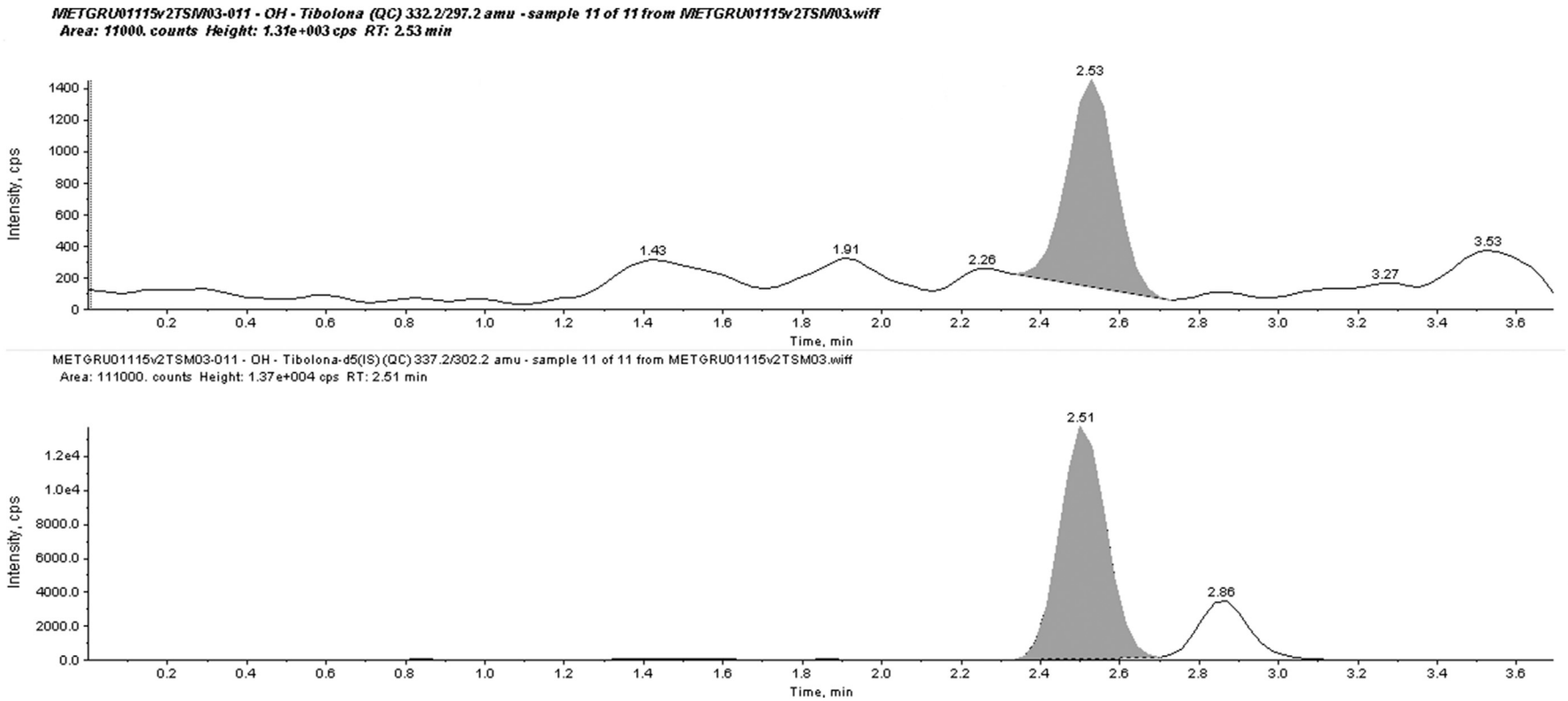

Fig. 6. Chromatogram at the limit of quantification (LOQ) (top channel: 3α-hydroxytibolone/bottom channel: internal standard).

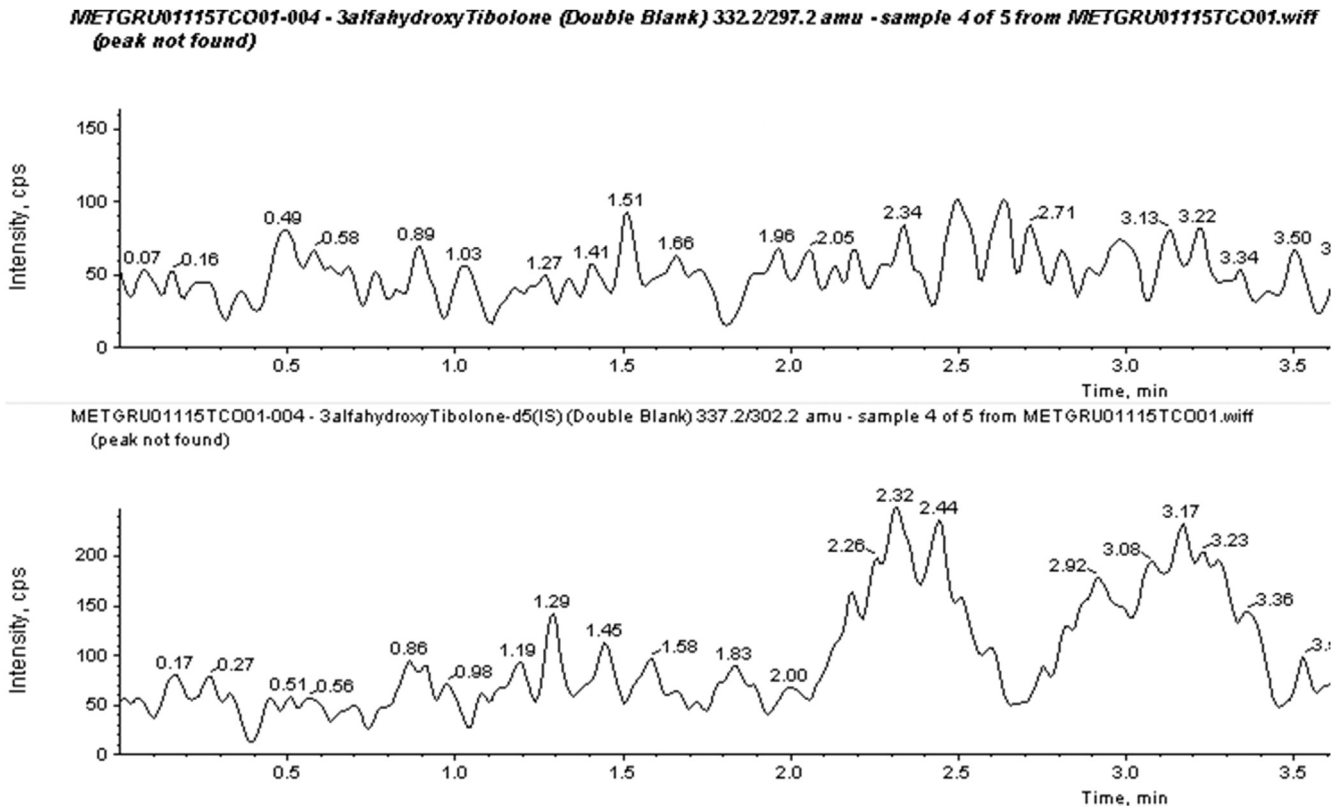

Fig. 7. Carry-over evaluation: chromatogram at blank normal plasma run after an injection of a high concentration quality control (QCH) sample (top channel: 3α-hydroxytibolone/ bottom channel: internal standard).

Table 2
Pharmacokinetic parameters after the administration of both tibolone formulations.

	Libiam™	Livolon
C_{max} (ng/mL)		
Mean	16.91	17.57
Range	7.92–31.70	5.78–37.50
T_{max} (h)		
Mean	1.61	1.48
Range	0.50–3.00	0.50–3.33
$AUC_{(0\text{-last})}$ (ng.h/mL)		
Mean	57.84	56.92
Range	19.64–93.79	24.18–101.01
$AUC_{(0\text{-inf})}$ (ng.h/mL)		
Mean	67.60	66.95
Range	22.12–110.38	27.87–114.98
k_e (h^{-1})		
Mean	0.15	0.14
Range	0.07–0.64	0.06–0.59
$t_{1/2}$ (h)		
Mean	5.40	5.94
Range	1.09–9.35	1.17–12.04

MS/MS methods employed for the determination of 3α-hydroxytibolone so far [20–23]. In contrast to our method, all the liquid chromatographic techniques mentioned employed *p*-toluenesulfonyl isocyanate derivatization to enhance the ionization process because the ionization efficiencies of most steroids are relatively low due to their apolar structure [24,25]. Previous attempts to develop LC-MS or LC-MS/MS assays for 3α-hydroxytibolone quantification without derivatization shown practically no response in negative ionization mode and low in positive ionization mode using either atmospheric pressure chemical ionization or electrospray ionization [20,23].

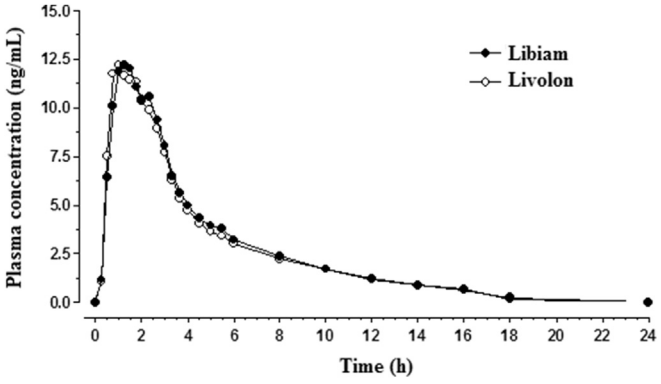

Fig. 8. 3α-hydroxytibolone plasma mean concentrations versus time profiles of each tibolone formulation.

Table 3
Summary of statistical analysis of C_{max}, $AUC_{(0\text{-last})}$ and $AUC_{(0\text{-inf})}$ individual test/ reference ratios.

	Ratio (%)	90% CI lower limit	90% CI upper limit
C_{max}	104.26	97.48	111.51
$AUC_{(0-last)}$	99.20	95.35	103.20
$AUC_{(0-inf)}$	100.07	96.42	103.86

Despite their apolar structure, steroids can sometimes be ionized with different ionization techniques using the adduct formation approach [26,27]; the analyte (3α-hydroxytibolone) and I.S. (3α-hydroxytibolone-d5) were detected mainly as ammonium adducts $[M + NH_4]^+$ (m/z 332.2 and m/z 337.4, respectively) that were

Table 4
Main data of the liquid chromatographic methods for 3α-hydroxytibolone quantification in human plasma.

	Zuo [20] LC-MS	Ban [21] LC-MS/MS	Shinde [22] UPLC-MS/MS	Cardoso [23] LC-MS/MS	Present method LC-MS/MS
Tibolone dose	2.5 mg	5.0 mg	2.5 mg	2.5 mg	3.75 mg
Internal standard	betamethasone	3α–OH–tibolone-d6	3α–OH–tibolone-d3	cyproterone	3α–OH–tibolone-d5
Plasma volume	1000 μL	200 μL	500 μL	250 μL	900 μL
Extraction technique	Liquid-liquid (ethyl acetate)	Liquid-liquid (ethyl acetate)	Liquid-liquid (ethyl acetate)	Liquid-liquid (chlorobutane)	Liquid-liquid (ethyl ether/hexane and ammonium hydroxide/water)
	Residue redissolved with acetonitrile	Residue redissolved with acetonitrile and sodium sulfate Followed by SPE (acetonitrile/water) Residue redissolved with mobile phase	Residue redissolved with acetonitrile	Residue redissolved with acetonitrile	Residue redissolved with mobile phase
Derivatization	*p*-toluenesulfonyl isocyanate	*p*-toluenesulfonyl isocyanate	*p*-toluenesulfonyl isocyanate	*p*-toluenesulfonyl isocyanate	None
Injection volume	80 μL	5 μL	10 μL	10 μL	20 μL
Mobile phase	Gradient elution (ammonium acetate/water and methanol)	Gradient elution (formic acid/water and methanol/water)	Isocratic elution (methanol and ammonium acetate/water)	Isocratic elution (acetonitrile/water)	Isocratic elution (acetonitrile/water and sodium hydroxide)
Flow rate	800 μL/min	250 μL/min	220 μL/min	800 μL/min	1150 μL/min
Column specifications	200 × 4.6 mm (5 μm)	50 × 2.0 mm (3 μm)	100 × 2.1 mm (1.7 μm)	100 × 4.6 mm (1.8 μm)	150 × 4.6 mm (5 μm)
Retention times (analyte and I.S.)	Approximately 6 and 5 min	Approximately 5.5 and 5.3 min	Approximately 3.2 and 3.1 min	Approximately 3.4 and 2.9 min	Approximately 2.5 and 2.5 min
Ionization process	ESI	ESI	ESI	APCI	ESI
Monitoring mode	SIM	SRM	MRM	SRM	MRM
LOQ	0.1 ng/mL	0.2 ng/mL	0.1 ng/mL	0.1 ng/mL	1 ng/mL

chosen as precursor ions for the MS/MS analysis. Signals were inadequate without the ammonium hydroxide additive. In addition, a higher tibolone dose (3.75 mg) and a larger plasma volume (900 μL) in comparison with the other liquid chromatographic methods, resulted in adequate analyte concentration in the electrosprayed solution. Lastly, the use of acetonitrile (solvent with low surface tension) in the mobile phase helped to achieve reproducible electrospray and the total flow rate was split in a 2:3 ratio to prevent analyte saturation in the ESI process. The injection volume was able to retain the chromatographic performance of column even after a large number of injections. Although a larger volume of plasma was used, no matrix effect or carry-over was observed and the hematological parameters of the volunteers showed no relevant alterations at the end of the study. Sufficient specificity was achieved by efficient chromatographic separation (relatively long column) and multiple reaction monitoring (MRM).

Furthermore, derivatization is a time consuming procedure that has some disadvantages including decreased precision due to the added derivatization steps and possible hydrolysis of conjugates which would affect the accuracy [28]. In addition, *p*-toluenesulfonyl isocyanate is a costly reagent with well-known health hazards (respiratory sensitisation, skin corrosion and serious eye irritation) [29,30]. Finally, considering all the gas and liquid chromatographic methods previously described, the LOQ ranged between 0.02 and 0.5 ng/mL. The LOQ (1 ng/mL) was adequate for the bioequivalence assay, without derivatization or expensive UPLC equipment, and the shortest retention times for 3α-hydroxytibolone and I.S. on average makes it suitable for high sample throughput studies.

5. Conclusion

The novel HPLC-MS/MS method described here for 3α-hydroxytibolone quantification in human plasma satisfies the requirements of sensitivity, specificity and high sample throughput for bioequivalence studies. Since the 90% CI for C_{max}, $AUC_{(0\text{-last})}$ and $AUC_{(0\text{-inf})}$ individual test/reference ratios were within the 80–125% interval indicative of bioequivalence proposed by A.N.V.I.S.A. and F.D.A., it is concluded that Livolon (1.25 mg tablet) provided by Biolab Sanus Farmacêutica (Brazil) is bioequivalent to Libiam™ (1.25 mg tablet) produced by Libbs Farmacêutica (Brazil), with regards to both rate and extent of absorption.

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