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Identification, Synthesis and Characterization of Process Related Impurity of Male Hormone Testosterone Undecanoate

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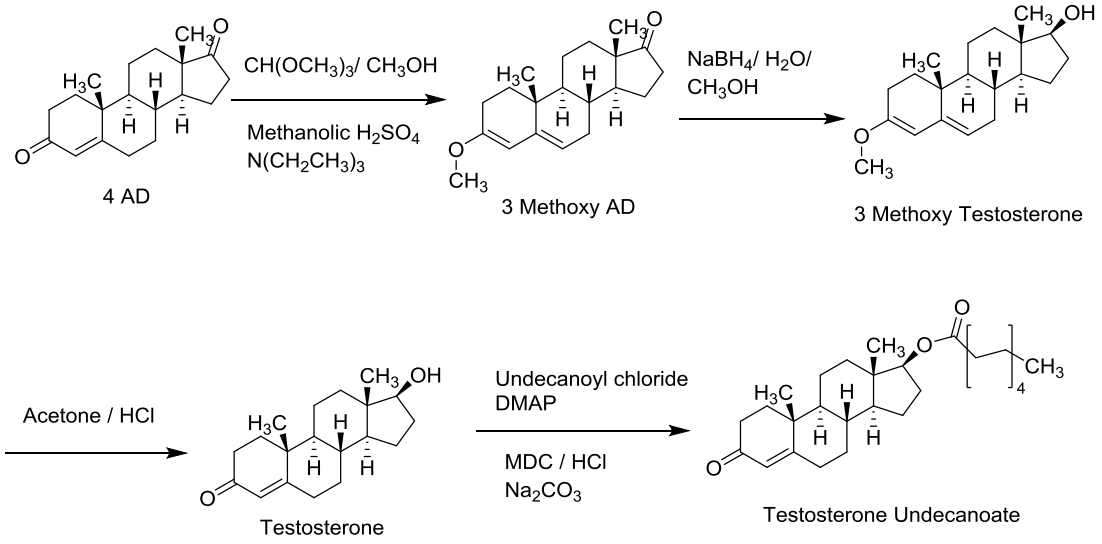
ABSTRACT

An unknown impurity observed in API testosterone undecanote was identified, synthesized and characterized using modern analytical techniques. Liquid chromatography–mass spectrometry (LC-MS) data revealed that the molecular weight of the impurity is two protons less than the API indicating presence of one additional double bond in the structure. Further the position of the double bond was confirmed by its synthesis. It is found to be the process related impurity.

Keywords: Testosterone undecanoate, 4-androstene-3,17-dione (4-AD), 3-methoxy androsta-4,9(11)-diene-17-one, Trimethyl ortho formate, Androsta-3,5,9(11)-triene-17-ol

INTRODUCTION

Testosterone is the primary male sex hormone and an anabolic steroid. In men, testosterone plays a key role in the development of male reproductive tissues such as the testis and prostate, as well as promoting secondary sexual characteristics such as increased muscle and bone mass and the growth of body hair [1]. Testosterone is first reported in 1935 [2]. Testosterone undecanoate is an androgen and anabolic steroid and a testosterone ester [3-5]. Testosterone undecanoate was first reported in 1953 [6]. It is used in androgen replacement therapy primarily for the treatment of male hypogonadism or as hormone replacement therapy in transgender men and has also been investigated for use as a male contraceptive [7]. It is marketed under the brand names Aveed, Andriol, Androxon, Cernos Depot, Nebido etc.



Scheme 1: Fermentation product

The starting material of testosterone is 4-Androstene-3,17-dione (4-AD) which is a fermentation product. Testosterone was prepared by methylation [8] of keto group at C3 in 4-AD using trimethyl ortho formate followed by reduction with sodium borohydride [9] and deprotection in acidic medium.

It was converted to Testosterone undecanoate by reaction of testosterone (1 Equation) with undecanoyl chloride (1, 2 Equation) in pyridine (1.52 Vol, 10.88 Equation) as base in ethyl acetate (12 Vol) as solvent at 20-25°C (Scheme 1). Reaction monitored by TLC (Hexane: Ethyl acetate; 8:2). The product was further purified by charcoal treatment in organic solvent.

In the present study it is observed that there is one unknown impurity appearing at 0.97 RRT showing $[M+H]^+$ ion peak at m/z 455 was identified in LCMS. The content of unknown impurity was in the range of 0.4% in the crude sample of the Testosterone Undecanoate (TUD). The purification of TUD leads to the loss in the yield. The objective of the present study was to identify the impurity which will get the insight or idea of source of the impurity and further helped to reduce well below acceptable limits.

EXPERIMENTAL

Materials and reagents

Samples of testosterone undecanoate were obtained from Center for Research & development, Ipca Laboratories Ltd. (Nandesari, Baroda, India). HPLC grade ACN, KH_2PO_4, KOH, CH_3COONH_4 was purchased from Merck India Limited (Mumbai, India). Deionized water was prepared using MilliQ plus purification system (Millipore, Bradford, MA, USA).

Mass spectrometry

Testosterone undecanoate sample (R&D/CRD/TUD/07) solution was subjected to mass spectrometer to find out the molecular mass of unknown impurity at RRT about 0.97, keeping mass range 115-700 Dalton. The liquid chromatography-heated electrospray ionization-tandem mass spectrometry (LC-HESI-MS/MS) analysis was carried out on Q Exactive (Thermo Scientific, Waltham, MA, United States) orbitrap mass spectrometer was used to achieve high-resolution accurate mass spectral data. The LC unit was consisted of an Ultimate 3000 quaternary gradient pump with a degasser and auto sampler. A Spherisorb C8 column (250 × 4.6 mm i.d., 5 µm particles) (Waters, Milford, MA, USA) was used for chromatographic separations. The mobile phase composed of 0.77 g ammonium acetate dissolved in 1000 ml of water (A) and acetonitrile (B) in a gradient mode (T_{min}/A:B; T_0/94:06; T_4/94:06; T_{45}/70:30; T_{50}/94:06). The flow rate was set to 1.0 ml per minute with UV detector wavelength was fixed at 210 nm. The sample solution (500 ppm) was prepared in mobile phase and 10 µl was injected. Mass parameters were set as; spray voltage was kept at 4.0 kV and capillary temperature at 320°C. Nitrogen was used as both sheath and auxiliary gas. Mass range was kept at m/z 100-1000. MS/MS studies were carried out by maintaining normalized collision energy at about 15% with the mass range m/z 50-750.

NMR spectroscopy

The ^1H and ^{13}C measurements were recorded on a Bruker AVANCE 400 NMR spectrometer (Fallanden, Switzerland) instrument at 300 K. The ^1H chemical shift values were reported on the δ scale in ppm relative to tetramethyl silane (TMS).

RESULTS AND DISCUSSION

Detection of impurity by HPLC

In the HPLC RS chromatogram of crude and pure Testosterone Undecanoate (TUD) showed one unknown impurity, which appears at 0.97 RRT? An impurity was present in the range of 0.08% levels in pure samples of TUD. This impurity does not correspond to any of the known impurities such as precursors or intermediates of TUD. This impurity was named as 0.97 RRT impurity. A typical HPLC chromatogram depicted below in Figure 1.

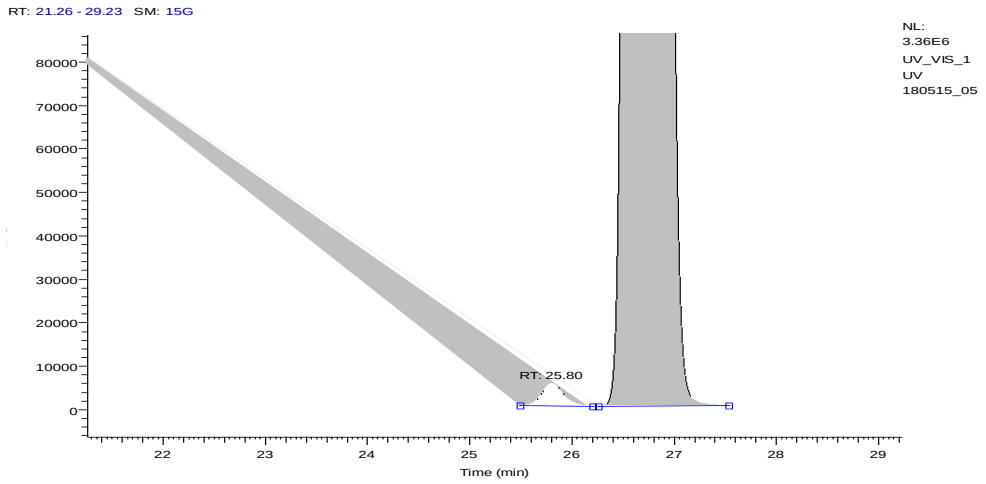


Figure 1: HPLC chromatogram

LC-MS and MS/MS

Since mobile phase used was not compatible for LC-MS, a new LC-MS compatible method was developed as given in experimental Section. MS/MS–MS spectrum was recorded in both positive and negative ion mode. However, positive ion mode produced most intense and stable MS and MS/MS spectrum. The positive ion HESI/MS spectrum of 0.97 RRT impurities.

An impurity appearing at RRT 0.97 showed [M+H]+ ion peak at m/z 455.3513 and MS/MS analysis gave fragment at m/z 269.1895, 251.17978, 173.1320. LC/MS and MS/MS of the product and 0.97 RRT impurity are given in the Figures 2A and 2B.

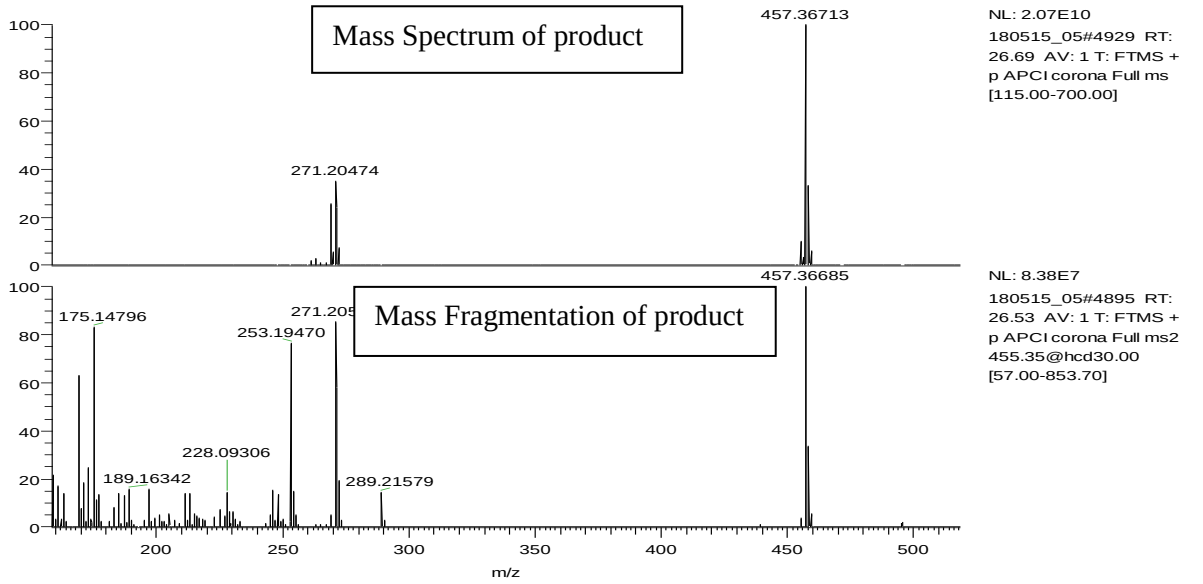


Figure 2A: LC/MS and MS/MS

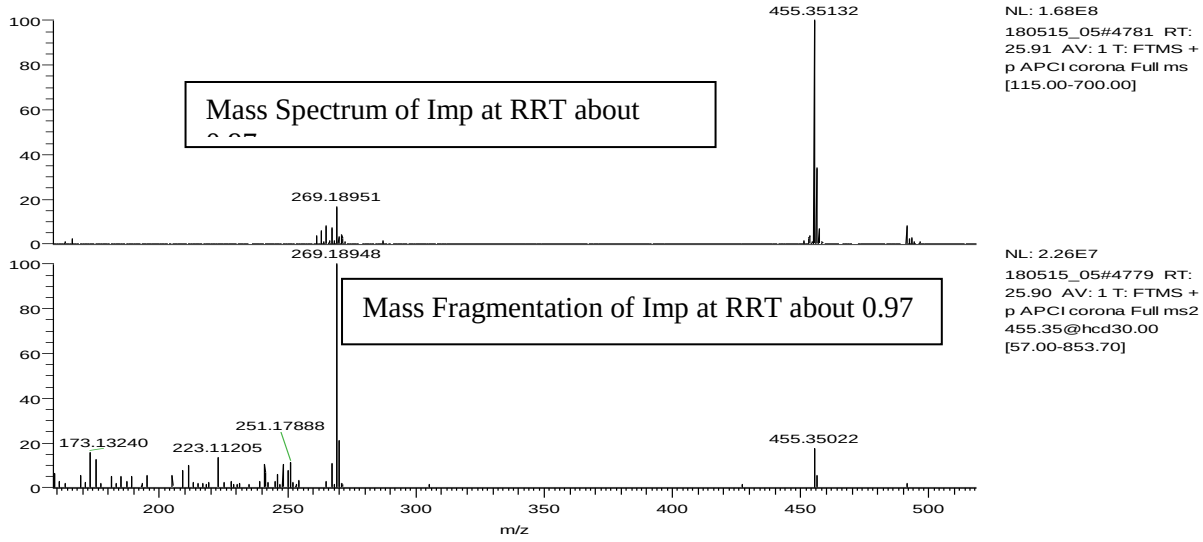


Figure 2B: LC/MS and MS/MS

Structure elucidation

In the positive mode m/z of RRT 0.97 impurity is 455.3513. The results of LC-MS m/z (M+H) are depicted in Table 1.

Table 1: LC-MS data for product and 0.97 RRT impurity

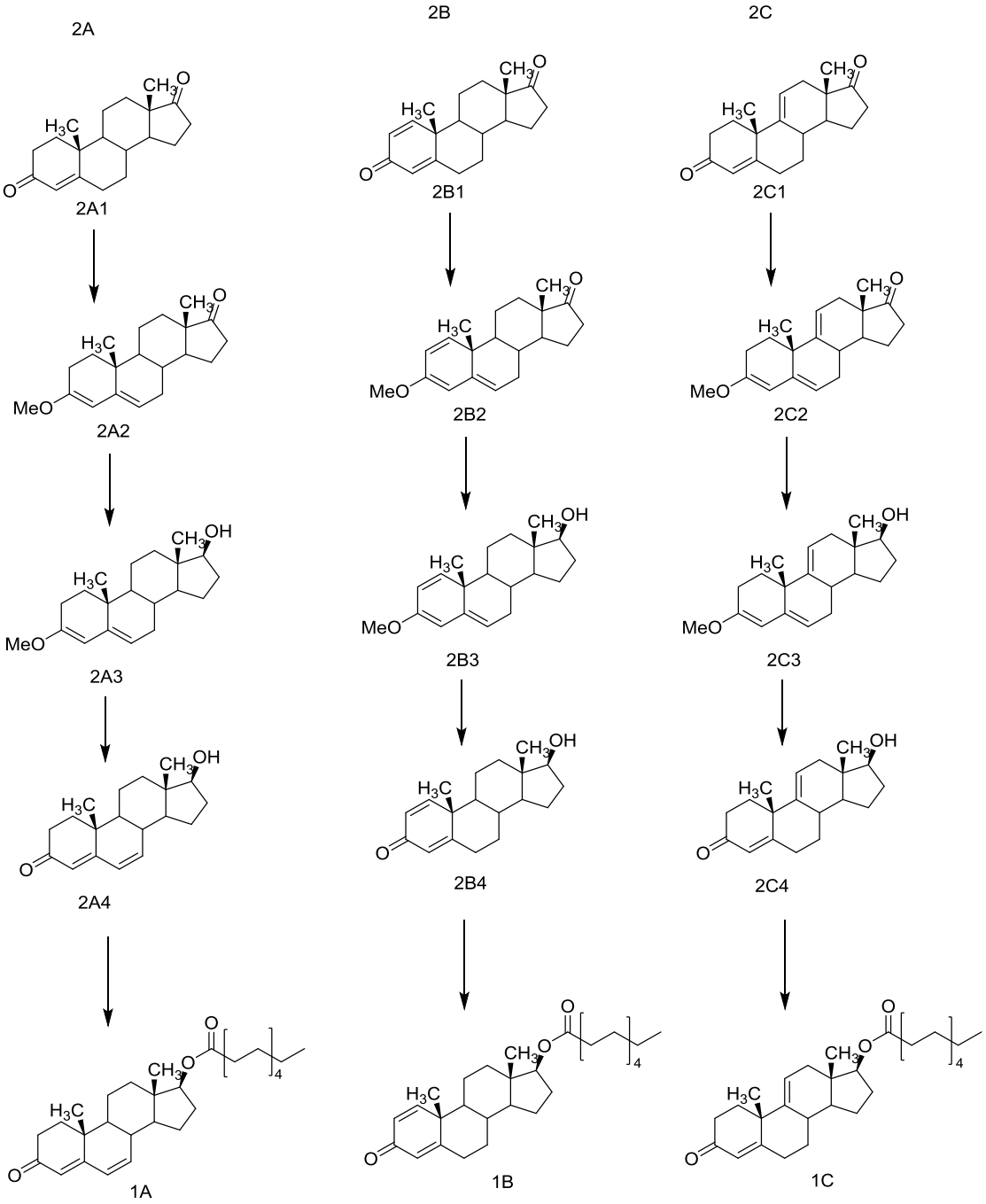
RT	m/z(M+H)	Molecular ion peak	MS/MS
25.8	455.3513	Impurity	269.1895, 251.1797, 173.1320
26.68	457.3669	Product peak (TUD)	271.2047,253.19443,175.1478

Thus as per the MS results the molecular weight of the unknown impurity is 454 indicating there are two proton less than that of TUD (456) which further indicate presence of two double bonds in the steroid structure i.e., one extra double bond, apart from double bond at 4 position. Also the RT of the impurity (RRT 0.97) is very close to the TUD peak which suggests that the impurity may be structurally related to TUD.

An attempt was made to isolate the impurity from TUD samples, however, the content was too less to carry out isolation by preparative chromatography. The separation of the product and impurity was not possible by flash chromatography. An attempt was also made to enrich the impurity from mother liquor, however the content was insufficient even in ML and thus impossible to isolate by any of the above techniques.

Thus it was thought that the origin of an impurity under study must be in the starting material. The starting material used for the preparation of TUD is 4-Androsten-3,17-dione (4-AD). There are no oxidative conditions in the reaction sequence of TUD preparation from 4AD (Scheme 1). This supports the fact that the origin of the 0.97 RRT impurity lies in the starting material.

These are process related impurities and can be originated from their corresponding precursors present in the starting material i.e., 4-Androstene-3,17-dione (4-AD). 4-AD is synthesized by fermentation process. The probable formation of these impurities is given in the Scheme 2.



Scheme 2: Possible method of preparation of proposed structures of impurities

The RS analysis (HPLC) of 4-AD showed, one unknown impurity in the range of 0.02-0.06% at 0.98 RRT. The HPLC chromatogram is given in Figure 3.

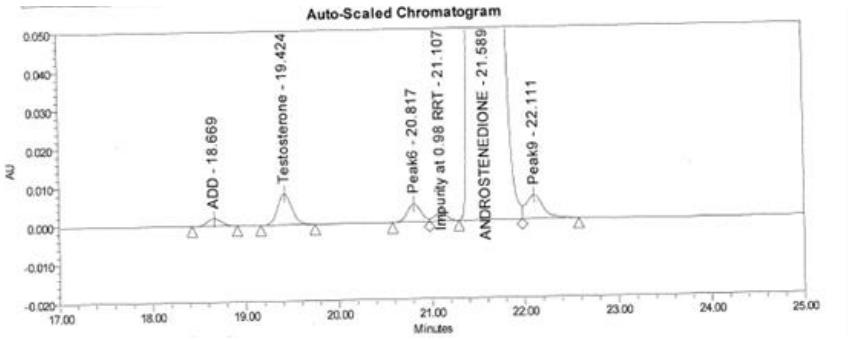
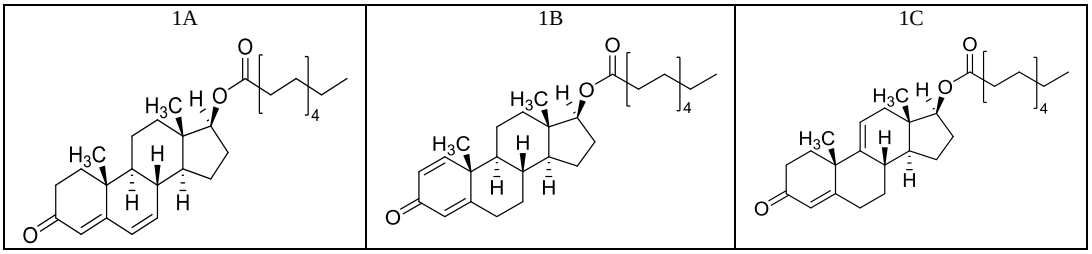
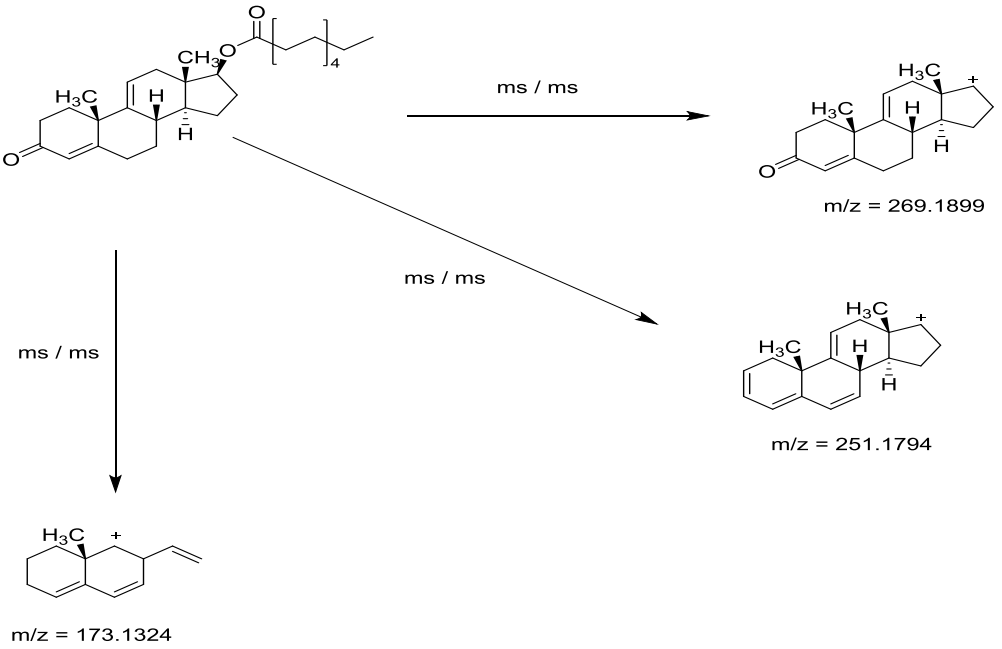


Figure 3: Since the unknown impurity in 4-AD is negligible it is difficult to separate by prep HPLC

Considering above discussion three possible structures for this impurity which are given below (1A, 1B and 1C)



An impurity 1A is Δ-6 testosterone undecanoate impurity which may be formed during preparation of testosterone; however Δ-6 testosterone impurity is controlled in testosterone stage and not present in testosterone. Thus this possibility is ruled out. An impurity 1B, was prepared from androsta 1,4-diene-3,17-dione (ADD) however it appears at different retention time. Thus 2C was prepared from Androsta-4,9(11)-diene-3,17-dione (2C1) the RRT of which matched well with an impurity under study (RRT 0.97) and also it is confirmed by spike study. It was synthesized and the structure was confirmed by MS and NMR analysis. Probable fragmentation is given in Scheme 3.

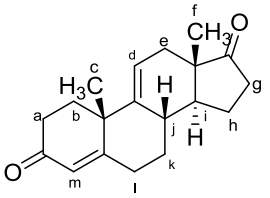
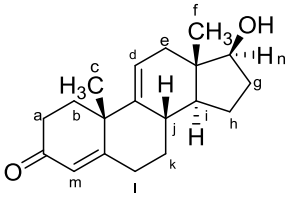
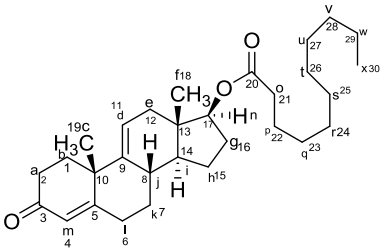


Scheme 3: Probable fragmentation of an impurity (0.97RRT) m/z = 454.3447

Synthesis of impurity and purification by combiflash

Preparation of 3-methoxy androsta-4, 9(11)-diene-17-one (1C): RRT 0.97 Impurity was prepared by following 2C reaction sequence given in Scheme 2. The numbering is given in the Table 2.

Table 2: Numbering of the steroid molecules in the present study is given below

S. No.	Name	Structure
1.	Androsta-4,9(11)-diene-3,17-dione (diene impurity: 2C1) & 3-methoxy-androsta-3,5,9(11)-triene-17-One (2C2)	
2.	Androsta-4,9(11)-diene-17-Ol (9(11)-dehydro testosterone: (2C4)	
3.	Impurity 0.97 RRT(1C):	

Androsta-4,9(11)-diene-3,17-dione (diene impurity: 2C1)

Diene (2C1) was prepared by known method [10]. 9-alpha-hydroxyandrost-4-ene 3,17-dione (3.02 g) was stirred with 150 ml of dry benzene and 6.94 ml of boron trifluoride acetic acid complex was added. The mixture was refluxed for 30 min and the reaction mass was cooled to room temperature (30-35°C). Water was added and stirred for 1 h. Androsta-4,9(11)-diene-3,17-dione (diene impurity: 2C1) was isolated by separating and evaporating organic layer to dryness. Yield 2.93 g 97%. ^{13}C NMR of showed two carbonyls 17°C and 3°C at 221.1 and 199.1 ppm respectively; 5°C at 169.1 ppm; 9°C 145.2 ppm; 4°C at 124.2 ppm; 11°C at 118.1 ppm; 14°C at 48 ppm; 13°C at 45.8 ppm; 10°C at 41.1 ppm; 8°C at 36.9 ppm; 12°C at 36.2 ppm; 16°C at 34.2 ppm; 1°C at 33.8 ppm; 2°C at 33.4 ppm; 6°C at 32.6 ppm; 7°C at 31.1 ppm; 19°C at 26.2 ppm; 15°C at 22.7 ppm; 18°C at 13.9 ppm. ^{1}H NMR of the starting material showed *s* 3(H) f at 0.88, *s* 3(H) c at 1.36 ppm; *m* 16(H) a, b, e, g, h, i, j, k, l between 1.12-2.64; *m* 1(H), d between 5.55-5.57; *d* 1(H) m at 5.76. m/z $(M+H)^+$ is 285.1854.

Preparation of 3-methoxy androsta-3,6,9 (11)triene-17-one (2C2)

(2C1, 5 g, 1 Equation) was treated with trimethyl ortho formate (5 ml, 2.612 Equation) in methanol (37 ml) below 20°C followed by addition of methanolic H_2SO_4 (0.5 ml of 5%) maintaining the temperature around 20°C under nitrogen atmosphere. Reaction was monitored by TLC (Hexane: Ethyl acetate: 8:2). Water was added to reaction mass and pH was adjusted at 8 using triethyl amine (~0.2 ml). Methanolic H_2SO_4 (1.6 ml of 5%) was added to reaction mass at 20°C and maintained for 1-2 h. Finally androsta-3,5,9(11)-triene-17-ol (2C2) was isolated by adjusting pH 8 using triethyl amine (~0.8 ml) and filtration. Wet cake of 2C2 was dried under vacuum Yield: 4.88 g, 93%. ^{13}C NMR of 2C2 showed 17ºC carbonyl at 221.8 ppm; 3ºC at 155.6 ppm; 5ºC at 144.7 ppm; 9ºC 139.0 ppm; 6ºC at 117.1 ppm; 11ºC at 115.2 ppm; 4ºC at 98.3 ppm; 20ºC at 54.4 ppm; 14ºC at 49.9 ppm; 13ºC at 46.3 ppm; 10ºC at 37.2 ppm; 8ºC at 36.5 ppm; 12ºC at 33.4 ppm; 16ºC at 33.2 ppm; 1ºC at 32.3 ppm; 7ºC at 31.8 ppm; 2ºC at 27.2 ppm; 19ºC at 25.4 ppm; 15ºC at 22.9 ppm; 18ºC at 13.7 ppm. 1H NMR of 2C2 showed *s* 3(H) f at 0.904, *s* 3(H) c at 1.173 ppm; *s* 3(H) n at 3.605 ppm; *d* 1(H) d at 5.19 ppm; *d* 1(H) l 5.3; *m* 14(H) a, b, e, g, h, i, j, k between at 1.603-2.59. m/z $(M+H)^+$ is 299.2013.

Preparation of 3-methoxy androsta -3,5,9(11)trione-17 one (2C3): 2C2 (4.88 g) was reduced with sodium borohydride (0.5 g, 1.212 eq) using methanol (24 ml) as solvent. Reaction was monitored by TLC. After complete disappearance of 2C2 water (19.5 ml) was added to the reaction mass and product (2C3) was isolated by filtration. The wet product (wet wt 4.88 g) was taken further for deprotection, m/z $(M+H)^+$ is 301.2169.

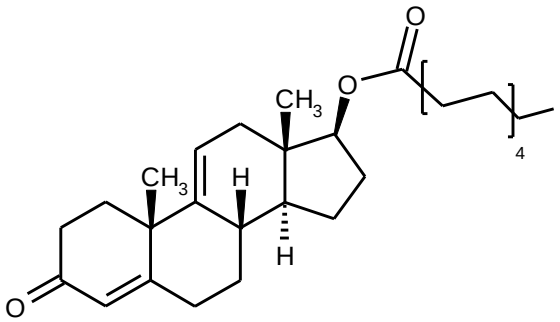
Preparation of 9(11)-dehydro testosterone (2C4): 2C3 (4.88 g) suspended in acetone (5 vol) and stirred with conc.HCl (0.1 ml) and heat reaction mass to 48 to 52°C for 1-2 h. Reaction was monitored by TLC. Reaction continued till disappearance of 2C3. Water (30 ml) was charged slowly to precipitate dehydro testosterone. Reaction mass was cooled to 25-30°C and stirred for ~3h. Reaction mass cooled to 0-5° for 1 h and filter. Impurity was purified by charcoal (4%) treatment in methanol acetone mixture (1:1) and precipitated using water. Product dried under vacuum at 50°C. Yield: 3.5 g, 70%. ^{13}C NMR showed 3C carbonyls at 199.6 ppm respectively; 5ºC at 170.3 ppm; 9ºC 144.7 ppm; 4ºC at 123.9 ppm; 11ºC at 118.7 ppm; 17ºC at 81.5 ppm; 14ºC at 47.6 ppm; 13ºC at 41.3 ppm; 10ºC at 41.1 ppm; 12ºC at 38.5 ppm; 8ºC at 37.5 ppm; 1ºC at 34.2 ppm; 2ºC at 33.8 ppm; 6ºC at 32.9 ppm; 16ºC at 31.7 ppm; 7ºC at 30.5 ppm; 19ºC at 26.1 ppm; 15ºC at 24.2; 18ºC 10.6 ppm. ^{1}H NMR of 2C4 showed *s* 3(H) f at 0.737 ppm; *m* 1(H) i between 1.02-1.064 ppm; *m* 1(H) j 1.181-1.254 ppm; *s* 3(H) c at 1.326 ppm; *m* 14(H) a, b, e, g, h, k, l between 1.345-2.546 ppm; *t* 1(H) n between 3.704-3.747 ppm; *d* 1(H) d between 5.502-5.516 ppm; s1(H), m at 5.72 ppm.

Preparation of 0.97 RRT Impurity: 1C was prepared by stirring 9(11)-dehydro testosterone (2C4, 2 g, 1 Equation) with undecanoyl chloride (2.3 g, 1.2 Equation) and pyridine (3.4 ml, 1.52 vol, 10.88 Equation) in ethyl acetate (12 Vol) as solvent at 20-25°C (Scheme 2) for about 1.5 h under nitrogen atmosphere. Reaction monitored by TLC. After complete consumption of 2C4 reaction was quenched with NaOH solution (2%).

Separate organic layer and washed with dil.HCl followed by water wash. The product was further purified by charcoal treatment in methanol and crystallized using methanol and water (Yield: 2.2 g, 77.46%). ^{13}C NMR showed 3C carbonyls 199.4 ppm; 20ºC at 173.9 ppm; 5ºC at 169.8 ppm; 9ºC 144.5 ppm; 4ºC at 124.0 ppm; 11ºC at 118.7 ppm; 17ºC at 82.2 ppm; 14ºC at 47.55 ppm; 13ºC at 41.15 ppm; 10ºC at 41.01 ppm; 12ºC at 38.8 ppm; 8ºC at 37.3 ppm; 2ºC at 34.23 ppm; 1ºC at 33.8 ppm; 21ºC at 32.84 ppm; 28ºC at 31.9 ppm; 6ºC at 31.7 ppm; 27ºC at 29.56 ppm; 23ºC at 29.47; 26ºC 29.44 ppm; 25ºC at 29.3; 24ºC at 29.27 ppm; 16ºC 29.14 ppm; 7ºC at 27.62; 22ºC 26.13 ppm; 19ºC at 25.12 ppm; 15ºC at 24.32 ppm; 29ºC at 22.7; 30ºC at14.2; 18ºC at 11.65 ppm. ^{1}H NMR spectrum showed *s* 3(H) f at 0.79 ppm; *m* 3(H), X at 0.86-0.90 ppm; *m* 37(H), a, b, c, e, h, i, j, k, l, m, o, p, q, r, s, t, u, v & w between 1.05-2.6 ppm; *m* 1(H) g at 4.68-4.73 ppm; 1(H), *m* 1(H), d at 5.50-5.51 ppm; *s* 1(H), n at 5.72 ppm. m/z $(M+H)^+$ is 455.3513. The target impurity 1C was finally purified by combiflash using hexane/ethyl acetate mixture (60:40).

CONCLUSION

Looking at mass and NMR data, the following structure of an impurity appearing at 0.97 RRT in Testosterone Undecanoate is confirmed.



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