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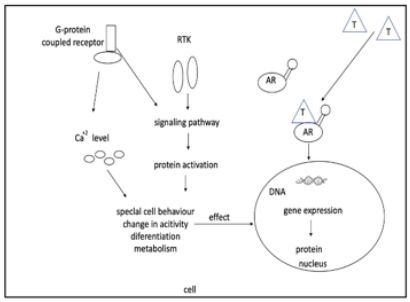
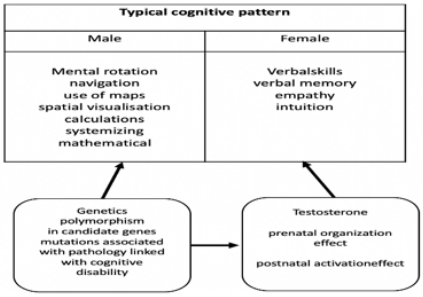
College of Science

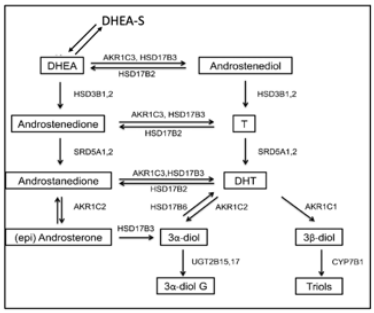
Department of Chemistry

LEVEL OF TESTOSTERON ESTERS IN CAMEL HAIR

Aysha Khalid Ali Alraeesi







April 2021

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Aysha Khalid Ali Alraeesi

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Chemistry

Under the Supervision of Professor Iltaf Shah

April 2021

Declaration of Original Work

I, Aysha Khalid Ali Alraeesi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Levels of Testosterone Esters in Camel Hair*" hereby solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Iltaf Shah, in the College of Science at UAEU. Other work has been published previously in the same area, but it does not form the basis for the award of any academic degree, diploma, or similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation, and/or publication of this thesis.

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Abstract

Doping and steroid use represent a serious threat to animal health and can even lead to their untimely and painful death. However, doping is an acute problem in today's animal racing world, particularly in camel racing. Testosterone and its ten esters (benzoate, valerate, isocaproate, hexahydrobenzoate, decanoate, undecanoate, laurate, enanthate, cypionate, and caproate) are of utmost importance, because when they are administered to animals it is difficult to measure them efficiently. The levels of testosterone and its esters in camels and other animals are typically determined using urine and blood tests. The aim of this study was to develop and validate a liquid chromatographic-mass spectrometric (LC-MS/MS) method to determine testosterone esters in camel hair, and to apply the validated method to determine testosterone esters in collected samples. To our knowledge, this is the first report of such a research. Camel hair samples were collected from 21 non-racing dromedary camels along with three racing camels in Al Ain, UAE; these were decontaminated, pulverized, sonicated, and extracted prior to analysis. An LC–MS/MS method was employed to determine the levels of testosterone esters in the hair samples. The levels of testosterone and its ten derivatives, along with the cortisol-D4 internal standard, were optimised for LC–MS/MS analysis; however, only testosterone along with its seven esters (namely benzoate, valerate, isocaproate, hexahydrobenzoate, decanoate, undecanoate and laurate) could be validated in camel hair. Only five testosterone esters could be determined in camel hair samples; the concentrations were obtained as 10.5–14.9 pg/mg for valerate (in three camels), 12.5–151.6 pg/mg for hexahydrobenzoate (in six camels), 4.8–32.1 pg/mg for laurate (in five camels), 5.1 pg/mg decanoate (in one camel), and 8.35–169 pg/mg for testosterone (in all 24 camels). Interestingly, the three racing camels displayed high concentrations of testosterone (59.2–169 pg/mg, all three camels), laurate (4.8–14.5 pg/mg, two camels), hexahydrobenzoate (116 pg/mg, one camel), decanoate (5.1 pg/mg, one camel) and valerate (11.7 pg/mg, one camel). This novel camel-hair test procedure is accurate, sensitive, rapid, and robust. The findings reported in this study could be significant to evaluate racing camels for suspected doping offenses. Further controlled testosterone-supplementation studies are required to evaluate individual esters

effects on camel health and diseases and on performance enhancement levels. This new hair test could promote further studies in doping control, toxicology, and pharmacology, as well as having other clinical applications relating to camel health, injury, and disease.

Keywords: Testosterone, testosterone esters, racing camels, hair, LC–MS/MS.

Title and Abstract (in Arabic)

مستويات التستوستيرون ايستر في شعر الجمل

الملخص

يعتبر تعاطي المنشطات والستيرويد تهديدًا خطيرًا لصحة الحيوانات، ويمكن أن يؤدي إلى موتها المبكر. ويعد تعاطي المنشطات مشكلة سائدة في عالم سباقات الحيوانات اليوم مثل سباقات الهجن، وتحديدا تعاطي التستوستيرون ومشتقاته. عادة ما يتم تحديد مستويات هرمون التستوستيرون ومشتقاته في الإبل باستخدام اختبار البول والدم. لكن الهدف من هذه الدراسة هو تطوير والـ تحقق من صحة طريقة قياس هرمون التستوستيرون ومشتقاته عن طريق تحليل شعر الجمل باستخدام جهاز الاستشراب السائل المزود بمقياس طيف الكتلة واستخدام الطريقة المعتمدة لتحديد مستوى التستوستيرون و مشتاقاته في عينات شعر الجمل التي تم جمعها. وهذه المرة الأولى الـ تي يتم فيها عمل هذا البحث. تم جمع 24عينه من شعر الجمل في مدينة العين بالإمارات العربية المتحدة وتم تطهيرها وسحقها استخلاصها قبل الـ تحليل، وتطوير هذه الطريقة والـ تحقق من صحتها باستخدام جهاز الاستشراب السائل المزود بمقياس طيف الكتلة تم تحسين التستوستيرون و مشتقاته العشرة جنبًا إلى جنب باستخدام جهاز الاستشراب السائل المزود بمقياس طيف الكتلة حيث تمكنا من كشف صحة 8 مشتقات فقط شعر الأبل. يمكن تحديد 5 مشتقات فقط من هرمون التستوستيرون في عينات شعر الإبل بتركيزات تتراوح بين 10.5-14.9 بيكوغرام / ملغ لـ Vularate (في 3 جمال)، و 12.5-151.6 بيكوغرام / ملغ لـ Hexahydrobenzoate (في 6 جمال)، 4.8 - 32.1 بيكوغرام / ملغ لوريت (في 5 جمال)، ديكانوفيت 5.1 بيكوغرام / ملغ (في جمل واحد)، و 8.35-169.6 بيكوغرام / ملغ لهرمون التستوستيرون تم العثور عليها في جميع الإبل 24. كان من المثير للاهتمام أن نلاحظ أن الإبل الثلاثة أظهرت تركيزات عالية من هرمون التستوستيرون (59.2-169.6 بيكوغرام / ملغم، جميع الإبل الثلاثة)، اللورات (4.8-14.5 بيكوغرام / ملغ، 2 .ما.)، 116.25) Hexahydrobenzoate بيكوغرام / ملغ، 1 جم .))، Decanovate 5.1) بيكوغرام / مجم ، 1 جمل) . هذا الاختبار الجديد لشعر الإبل د قيقة وحساس وسريع فعال. النتائج الواردة في هذه الدراسة يمكن أن تكلالا ذات لألايلا عالاقييلا الابل قبل السباق للاطلاع على المنشطات الموجودة. هناك حاجة إلى مزيد من الدراسات الخاضعة للرقابة بشأنلالاملات التستوستيرون لتقييم تأثير المشتقات على صحة الإبل ومدى تحسين أداء ...مل. يمكن أن يساعد اختبار الشعر الجديد هذا في إجراء مزيد من الدراسات في مكافحة المنشطات، ودراسات السموم، والدراسات الدوائية والتطبيقات السريرية الأخرى في صحة الإبل، وا لإلإاوا ،ةبلأمراض.

مفاهيم البحث الرئيسية: شعر ا لجمل، نواتج التستوستير ون، المنشطات.

Acknowledgements

This thesis could not have become a reality without the help and support of many individuals. I wish to express my gratitude towards my professor, Iltaf Shah, for helping me to expand my knowledge and develop my learning and research skills, as well as for supporting and guiding me. Additionally, I owe a deep sense of gratitude towards the members of Chemistry Department at the UAE University, who gave me the opportunity to conduct this incredible research and have made future research opportunities available to me. I am also thankful to my thesis committee, who assisted and encouraged me in conducting the research. Furthermore, I would like to thank my friend (Amir Sohail) who helped me finalize this project within the limited time frame. Besides this, I am thankful to the scholarship office officials in the Ministry of Presidential Affairs, for providing the funding needed to conduct the research

I want to express wholehearted gratitude towards my husband, who has helped me conduct the research by providing mental guidance and support; this helped me to understand my strengths and weakness and to conduct the research adequately. I am incredibly thankful to my parents for their sacrifices, care, prayers, and love, through which they have educated and prepared me for my future. It would have been impossible to complete my master's without their constant support and true care.

Dedication

To my beloved parents and family

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List of Abbreviations

AAS	Anabolic Androgenic Steroids
ACN	Acetonitrile
CID	Collision-Induced Dissociation
ESI	Electrospray Ionization
HPLC	High-Performance Liquid Chromatography
HPLC–MS/MS	High-Performance liquid Chromatography–Tandem Mass Spectrometry
IQ	Intelligence Quotient
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
MeOH	Methanol
MRM	Multiple Reaction Monitoring
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS	Mass Spectrometry
PEDs	Performance-Enhancing Drugs
QCH	Quality Control High
QCL	Quality Control Low
QCM	Quality Control Medium

RT	Retention Time
SD	Standard Deviation
SHBG	Sex-Hormone-Binding Globulin
TA	Testosterone Acetate
TB	Testosterone Benzoate
TC	Testosterone Cypionate
TD	Testosterone Decanoate
TE	Testosterone Enanthate
TI	Testosterone Isocaproate
TP	Testosterone Propionate
TPh	Testosterone Phenylpropionate
TU	Testosterone Undecanoate
UAE	United Arab Emirates
WADA	World Anti-Doping Agency

Chapter 1: Introduction

1.1 Overview of Thesis

Steroid misuse is an acute global problem involving both humans and animals [1]. Different types of drug therapy have been adopted to treat animals and enhance their performance, or to treat an injured animal such that it is more likely to win a competition [2].

In particular, steroids and anabolic-androgenic steroids (AAS) are used by animal—especially camel—owners to enhance the competitiveness of the camels. Steroid usage has also expanded from elite athletic circles into high-school athletic programs. Steroid use has been accused of providing an unfair advantage and damaging the true talent of a competitor. Performance-enhancement drugs are considered to be attractive by younger generations, and this may increase the usage of these drugs in the future [3]. Performance-enhancing drugs are considered unethical and a form of cheating; hence, they are illegal and have been prohibited by the World Anti-Doping Agency (WADA). In most countries, doping has been strictly prohibited as part of curbing the illegal use in the sporting world and promoting healthy competition. Doping has been prevalent for many years in sports, with no positive indication of being diminished [4].

Recent years have seen a significant increase in awareness and research into various aspects of doping. Awareness campaigns are necessary for educating current generations about the harmful effects of doping on society and the health of humans and animals.

1.2 Camel Racing

Camels have formed a part of the Bedouin lifestyle and culture for centuries. Camel racing is widely popular in Gulf countries; in Dubai, it is a huge attraction for both visitors from across the world and Emiratis. The peak season for camel racing falls between the months of October and April, on Friday and Saturday mornings. The largest racing track in Dubai is the Al Marmoom Camel Racetrack, which belongs to the Dubai Camel Racing Club. Special efforts have been made to maintain the core traditions of the game. Owners from across the United Arab Emirates (UAE) and Saudi Arabia are attracted to the race [5]. Thousands of dirhams are awarded to the winners, and other special prizes such as luxury cars are also given. The extravagant nature of the competition and the lucrative prizes available often compel the trainers to cheat. The camels are subjected to doping procedures, to increase their performance and to gain a competitive advantage. The sport has been considerably updated in recent years, to ensure fair competition [6]. Doping is generally illegal across the globe [7]. In the UAE specifically, the government has been very vocal about the eradication of doping. Rigid policies have been introduced to restrain doping practices in the region. Doping amounts to nothing less than animal abuse; it is a heinous crime to subject innocent animals to prohibited drugs. Three types of methods are used to dope camels: liniments, oral drugs, and injections. Drugs can be natural or synthesized depending on their origin and are used in various ways and on various levels. Certain drugs (e.g., ammonium chloride and furosemide) are used to treat medical conditions in animals; these are legal, and therapeutic use exemptions can be obtained for them [8]. A number of trainers and veterinarians have been charged for administering performance-enhancing drugs that they were committed to establishing fair competition

in upcoming events; they have reportedly made significant improvements in the anti-doping testing procedures. Strict vigilance is being maintained to (PEDs) to animals. Recently, the organizers of the Dubai Camel Racing Festival announced mitigate the likelihood of cheating in the competitions. The UAE has undoubtedly taken rigorous steps to prevent the cruel practice of animal doping. Continual developments have also been made towards accurate and robust testing and analysis procedures in the country [9]. Doping can be detected by testing hair, urine, and saliva samples of a person or animal. Anabolic steroids present in human hair have been detected using novel, highly specific, sensitive, and reliable liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods [10]. Hair analyses are used as a complementary test to blood and urine analyses. Recently, hair analysis has been implemented to identify chronic drug use or long-term drug doping [11]. Thus, hair represent the best method for identifying competition doping.

1.3 Hair Analysis

Hair analysis is a safer non-invasive test procedure than other methods [12] and hence no harm is done to the animals. Hair sample testing does not require experts, and a non-specialist can more easily conduct hair tests than blood sample procedures, for which numerous instruments and devices are used. Hair samples are easy to store and transport, and the sample quality does not rapidly degrade [13].

Hair analysis presents a negligible risk of infection during collection, reducing the risks of cross-contamination and tampering. Hair analysis is currently being used to detect drug usage, and it is also gaining attention for anti-doping controls. Furthermore, it provides a larger time period for surveillance. The average hair growth is 1–1.5 cm per month (in

humans and animals), and the larger strands contain more retrospective information regarding drug intake than the short-term-usage information found in urine or blood samples. Occasionally, results that detect and confirm steroid usage can be false positives, owing to the consumption of foods containing undeclared substances.

Animal owners use steroids to increase the animal strength, body mass, and aggressiveness; they can also help to shorten the recovery time between different races. It is known that the use of steroids during or before a competition is illegal and carries the risk of banning. In 1974, the International Olympic Committee added doping agents to the list of prohibited substances, owing to their effects on athlete performances [4]. In sports—where steroid usage is popular among animal owners—urine specimen analyses fail to clearly distinguish between single exposures or chronic use. Hair analysis provides a larger detection window; thus, it offers an advantage over other tests in that it can identify drug users who try to cheat the test by diluting their urine sample or abstaining from drugs for a few days before the test. Hence, hair testing or analysis can be seen as a more modern method that offers significant advantages such as increased accuracy and tamper-resistance. Thus, hair analysis is beneficial and presents a negligible risk of infection, cross-contamination, or tampering [14].

1.4 Anabolic Steroids

Anabolic steroids (more correctly referred to as anabolic-androgenic steroids) are steroidal androgens that contain normal androgens (e.g., testosterone) along with synthetic androgens, which are physically connected and have the same effects as testosterone.

Diverse side effects can arise when anabolic steroids are distorted, extending from minor problems up to dangerous or even deadly ones. Most effects are rescindable once the user stops applying the drugs. The side effects of anabolic steroids can include severe acne, hair loss, oily skin and hair, liver diseases (e.g., cysts and liver tumors), heart diseases (e.g., strokes or heart attacks), Q1A diseases altered moods, increased violence, suicidal tendencies, depression, and changes to fat and other blood phosphatides. Anabolic steroids can render the user both psychologically and physically reliant on the drugs, and constant use can even produce the opposite effects to those intended, as well as physical withdrawal symptoms such as mood swings, exhaustion, agitation, loss of appetite, insomnia, reduced sex drive, and steroid addiction. Simple withdrawal can lead to suicide as well as depression. Depressive symptoms can persevere for one year after halting anabolic steroid usage. Some people who inject anabolic steroids may lack sterile injection methods, or they may share unclean needles with other people; this places them at risk of life-endangering viral contagions (e.g., HIV and Hepatitis B and C). Furthermore, animal models indicate that anabolic steroids destroy the immune system; this can exacerbate infections [15].

One motive for continuing anabolic steroid usage after its ban is that it can allow the user to outperform competitors who abstain. Besides this, it may influence the methods used to win sports tournaments and give an unfair advantage to people who do not misuse medicines. Furthermore, steroidal dietary supplements can be converted into testosterone or other androgenic mixtures in the body. Over-the-counter steroidal nutritional supplements (e.g., androstenedione) and tetrahydrogestrinone are currently illegal. Clinical investigation reports demonstrate numerous severe side effects, in addition to

disturbing the normal levels of hormones in the body. The decrease in testosterone leads to reduced sperm formation and testicle sizes. Anabolic steroids can also perform hormone coordination, increasing the possibility of testicular cancer [16].

1.5 Formation of Testosterone in the Body

The testosterone hormone is synthesized by the gonads. In males, it is produced by the Leydig cells found in the testes; in females, it is produced by the ovaries. Despite being produced by both sexes, testosterone is an androgen and stimulates the development of male characteristics. It is produced in small quantities. Cholesterol acts as the source of key substances for testosterone formation, and the Leydig cells obtain these substances from cholesterol floating in the blood [16]. Sexual activity and sexual stimulation increase testosterone levels in the body; likewise, sustained sexual inactivity can cause them to drop. Low levels of testosterone in the male body can lead to erectile dysfunction.

1.6 Breakdown of Testosterone into Other Metabolites in the Body

Testosterone is primarily metabolized in the liver. Approximately 50% of the testosterone hormone is decomposed through conjugation and leads to the formation of testosterone sulfate and testosterone glucuronide via sulfotransferases and glucuronosyltransferases enzymes, respectively; 40% of the testosterone is metabolized into equal proportions etiocholanolone and 17-ketosteroids androsterone [17] with the help of 5α- and 5β-reductases, 3α-hydroxysteroid dehydrogenase, and 17β-HSD enzymes. Androsterone and etiocholanolone are glucuronidated and sulfated to a lesser extent, similar to testosterone. The hepatic metabolites and testosterone conjugates are released into the circulation system and finally excreted in the bile and urine.

A small fraction of the hormone (i.e., 2%) is expelled without a change in the urine [18]. During testosterone metabolism, the conversion of testosterone takes place in the liver via the hepatic 17-ketosteroid pathway. Inactive 5β-DHT and 5α-DHT are produced by the conversion of testosterone via 5β-reductase and 5α-reductase, respectively. Subsequently, 3α-etiocholanediol and 3α-androstanediol are converted into etiocholanolone and androsterone, respectively, by 17β-HSD and subsequently conjugated and excreted. A very small portion of the hormone (i.e., ~3%) is reconverted into androstenedione by 17β-HSD in the liver.

Like most hormones, the testosterone hormone is supplied to the targeted tissues via the blood. This transportation occurs within a specific bonded structure, in which the hormone binds with a particular plasma protein: sex-hormone-binding globulin (SHBG). Approximately 5–7% of the testosterone is converted into 5α-dihydrotestosterone (5α-DHT) via the 5α-reductase enzyme, for which the 5α-DHT circulation levels are ~10%, similar to those of testosterone [17]. Moreover, 0.3% of the hormone is converted into estradiol with the help of the aromatase enzyme as shown in Figure 1.

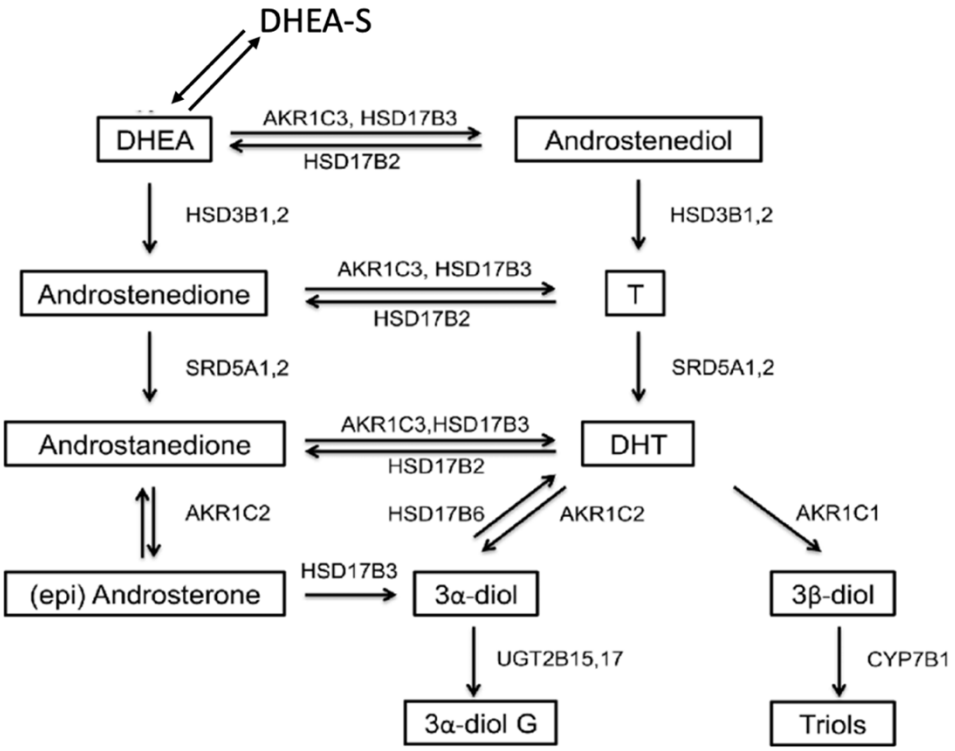

Figure 1: Chemical reaction and bonding [19]

1.7 Testosterone Esters Their Structures and Properties

1.7.1 Testosterone Hormone

Testosterone is a hormone that is generated by the human body. In men, it is largely produced by the testicles. It determines the emergence and growth of sexual characteristics in men. Besides this, it assists with muscle and bone accumulation. This study aims to analyze and discuss the chemical structure, effects, and endo\exogenous nature of testosterone.

The chemical structure of testosterone is $C_{19}H_{28}O_2$. Testosterone features 17 beta-hydroxy and three oxo groups, as well as unsaturated C-4-C-5 as shown in Figure 2. Testosterone

deficiency syndrome occurs when the body cannot generate sufficient testosterone [4]. This mostly affects older men; however, younger patients can be affected for many reasons.

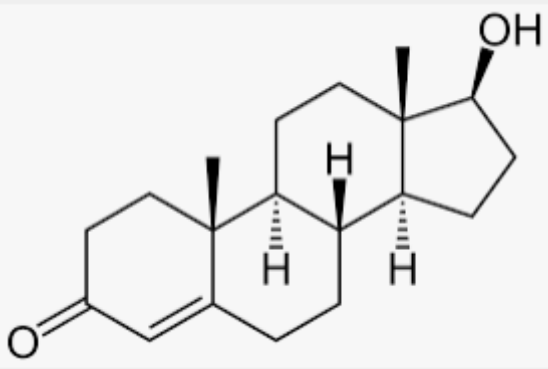

Figure 2: Chemical structure of testosterone [3].

1.7.2 Role of Testosterone

Testosterone is involved in the growth of muscle volume and strength. It stimulates the growth of neurotransmitters that promote tissue and growth hormone development. Approximately 6 weeks after conception, testosterone begins to shape the male genitals. Throughout puberty, increasing testosterone levels promote the development of the testicles, pubic hair, and penis [20]. It plays a vital role in behaviors associated with violence and supremacy. Testosterone-substitution therapy produces several possible side effects, including acne and breast extension. Testosterone regions may cause an insignificant skin annoyance. Testosterone enhances bone thickness and instructs the bone substance to produce red blood cells.

1.7.3 Endogenous and Exogenous Testosterone Supplementation

Exogenous testosterone supplementation reduces sperm production by reducing follicle-stimulating hormone (FSH) and luteinizing hormone (LH) which in turn affect the Sertoli cells in the testes where sperm are produced. In the testis, somatic Sertoli cells change over signals from testosterone and FSH and LH into the production of factors that are required by germ cells as they mature into spermatozoa. Endogenous imports and procedures are those that arise from within an organism, cell, or tissue [21].

Endogenous substances and processes differ from exogenous ones because endogenous originate from within a system, such as hormones, while exogenous are produced outside the individual such as synthetic drugs.

Testosterone and the aforementioned anabolic-androgenic steroids improve athletic performance. Many of the testosterone products approved for therapy nowadays are testosterone ester. Ester has multiple functions and is mixed with testosterone to extend its lifespan and bulk manipulability. When infused, pure testosterone has a short half-life, enduring in the body for just a few hours [22]. However, testosterone-induced physiological modifications may not frequently produce measurable changes in human performance. New data have led to the improvement of our physiological understanding of how testosterone management cooperates with exercise ability; this understanding can assist anti-doping efforts. Androgen treatment can affect polycythemia in sleep apnea. However, these effects can be harmful in men with negotiated cardiac functions. Short-term testosterone usage may also have cryogenic impacts, as exhibited by improved maximal bench-press power [20].

Testosterone therapy can help mitigate the impacts of hypogonadism; however, its effectiveness in older healthy men remains unclear.

Testosterone is the most prominent socializing androgen in the male body, and it performs a significant role in the diagnosis and identification of several diseases. Furthermore, hypogonadism has a prevalence of ~20%. A small quantity of socializing testosterone is transformed into estradiol, an estrogen metabolite. The ideal bio-accessible testosterone is produced through the division of testosterone.

1.8 Testosterone Esters Under Investigation

1.8.1 Testosterone Laurate

Testosterone laurate which is shown in Figure 3 is an ester of testosterone which is chemically synthesized in the lab. The chemical symbol is $C_{31}H_{50}O_3$. It is Endogenous and the half-life is 4.3 hours. It is used in the body as a treatment of breast cancer, osteoporosis, cachexia, and anemia. It can also cause increase in blood-clot risk, breast enlargement, development of prostate abnormality, fluid retention, oily skin, and acne. Testosterone laurate. deficiency can cause hypogonadism.

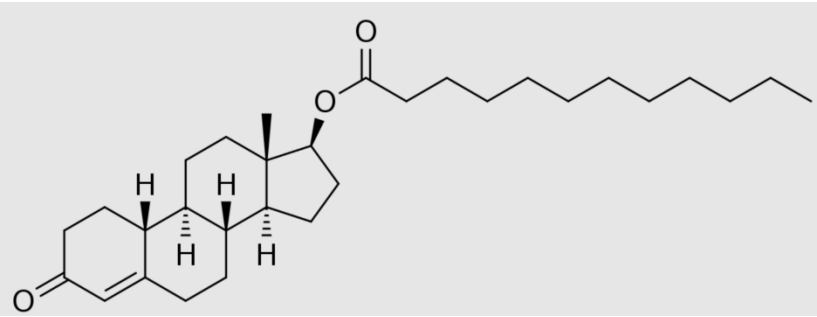

Figure 3: Structure of testosterone laurate [17].

1.8.2 Testosterone Undecanoate

Testosterone undecanoate which is shown in Figure 4 is an ester of testosterone which is chemically synthesized in the lab. The chemical symbol is $C_{30}H_{48}O_3$. It is Exogenous and the half-life is 33.9 days. It is used in the body as a replacement theory of androgens. It can also cause symptoms of masculinity, such as increase in sexual desire, voice change, abnormal hair growth, and acne. Testosterone undecanoate deficiency can cause Medication deficiency can lead to increased body fat, decreased body muscle mass, and increased bone fragility [17].

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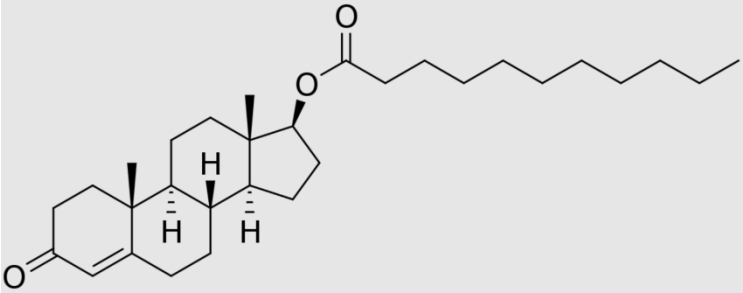


Figure 4: Structure of testosterone undecanoate [17].

1.8.3 Testosterone Isocaproate

Testosterone isocaproate which is shown in Figure 5 is an ester of testosterone which is chemically synthesized in the lab. The chemical symbol is $C_{25}H_{38}O_3$. It is exogenous and the half-life is 2 years. It is used to replace testosterone in males afflicted with primary and secondary hypogonadism. It can also cause weight gain, nervousness, irritability, and frequent erections. Testosterone isocaproate deficiency can cause causes numerous health

problems, including bone loss, depressive moods, tiredness, low sex drive, infertility, and impotence [17].

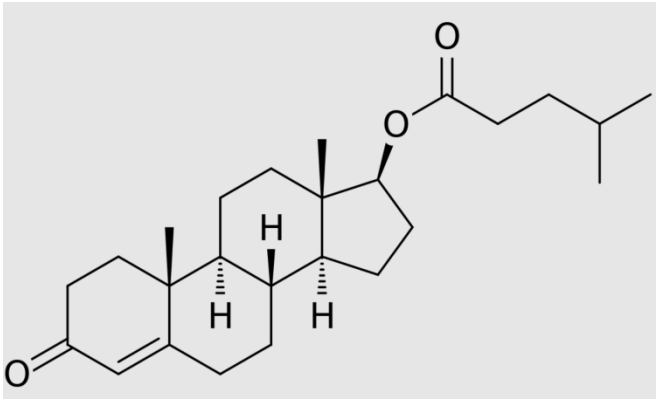


Figure 5: Structure of testosterone isocaproate [17].

1.8.4 Testosterone Enanthate

Testosterone enanthate which is shown in Figure 6 is an ester of testosterone that is chemically synthesized in the lab. The chemical symbol is $C_{26}H_{40}O_3$. The half-life is 7–9 days. It is used to replace theory of androgens. It can also cause Acne, hair loss, decreased or increased sexual interest, skin-color change, headache, vomiting, and nausea. Testosterone enanthate deficiency can causes erectile dysfunction, body fat increase, decrease in muscle or bone mass, and decreased sperm count [17].

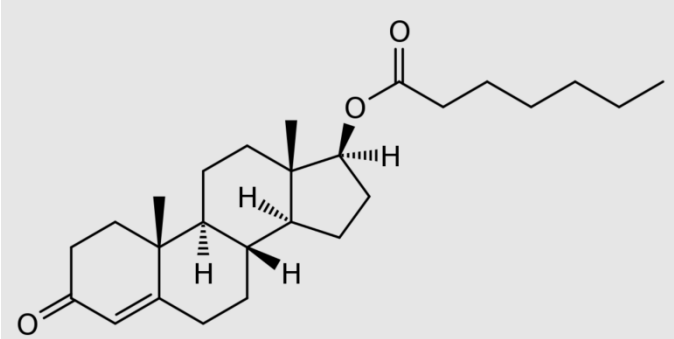


Figure 6: Structure of testosterone enanthate [17].

1.8.5 Testosterone Benzoate

Testosterone benzoate which is shown in Figure 7 is an ester of testosterone that is chemically synthesized in the lab. The chemical symbol is $C_{26}H_{32}O_3$. The half-life is 8 days. It is used as a prodrug testosterone. It can also cause pulmonary oil microemboli and anaphylaxis estosterone benzoate deficiency can cause erectile dysfunction, body fat increase, decrease in muscle or bone mass, and decreased sperm [17].

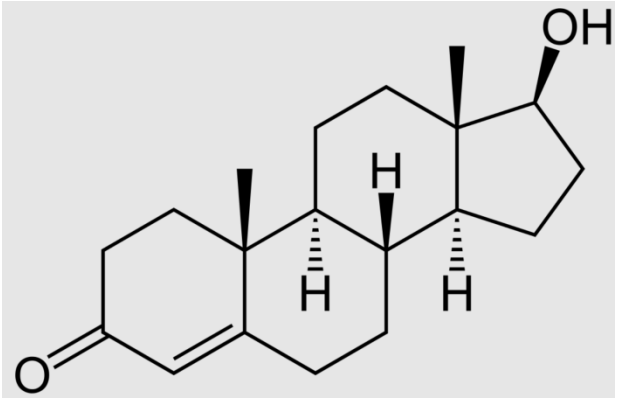


Figure 7: Structure of testosterone benzoate [17].

1.8.6 Testosterone Cypionate

Testosterone cypionate which is shown in Figure 8 is an ester of testosterone which is chemically synthesized in the lab. The chemical symbol is $C_{27}H_{40}O_3$. The half-life is 8 days. It is used to treat symptoms of hypogonadism in males. It can also cause acne, hair loss, decreased or increased sexual interest, skin-color change, headaches, vomiting, and nausea. Testosterone cypionate deficiency can cause fragile bones and increased body fat and muscle/bone mass [17].

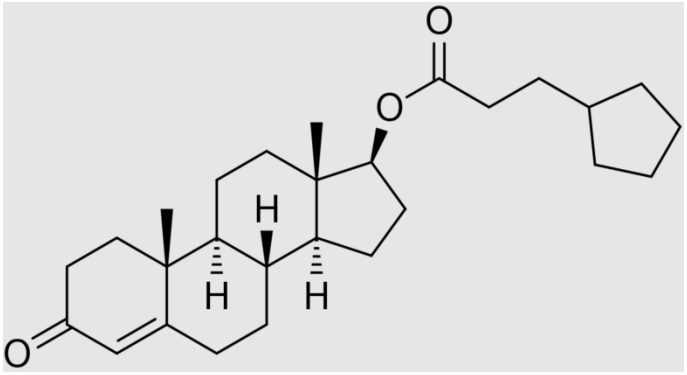


Figure 8: Structure of testosterone cypionate [17].

1.8.7 Testosterone Decanoate

Testosterone decanoate which is shown in Figure 9 is an ester of testosterone which is chemically synthesized in the lab. The chemical symbol is $C_{30}H_{48}O_3$. The half-life is 20.9 days; elimination half-time: 33.9 days. It is used to produce the requisite amount of testosterone within the male body. It can also cause aggression, insomnia, fatigue, increased estradiol, hypogonadism, and acne. Testosterone decanoate deficiency can cause fragile bones and increased body fat and muscle/bone mass [17].

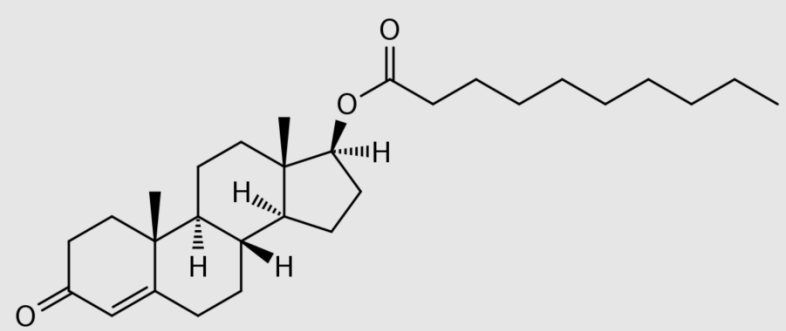


Figure 9: Structure of testosterone decanoate [17].

1.8.8 Testosterone Valerate

Testosterone valerate which is shown in Figure 10 is an ester of testosterone which is chemically synthesized in the lab. The chemical symbol is $C_{24}H_{36}O_3$. It is used to produce the requisite amount of testosterone within the male body. It can also cause acne, hair loss, decreased or increased sexual interest, skin-color change, headache, vomiting, and nausea. Testosterone valerate deficiency can cause erectile dysfunction, body fat increase, decrease in muscle/bone mass, and decreased sperm count [17].

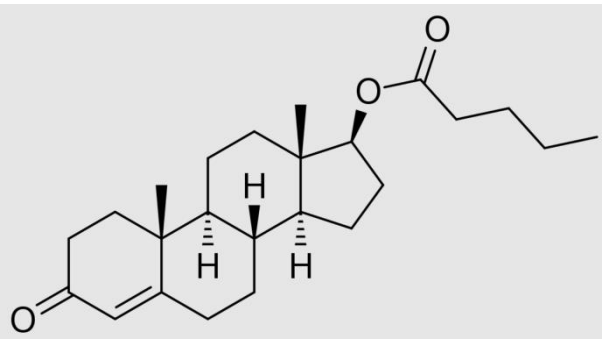


Figure 10: Structure of testosterone valerate [17].

1.8.9 Testosterone Caproate

Testosterone caproate which is shown in Figure 11 is an ester of testosterone which is chemically synthesized in the lab. The chemical symbol is $C_{25}H_{38}O_3$. It can be endogenous and exogenous. It is used in the replacement theory of androgens. It can also cause Symptoms of masculinity, including increased sexual desire, voice changes, abnormal hair growth, and acne. Testosterone caproate deficiency can cause Increased body fat, decreased body muscle mass, and increased bone fragility [17].

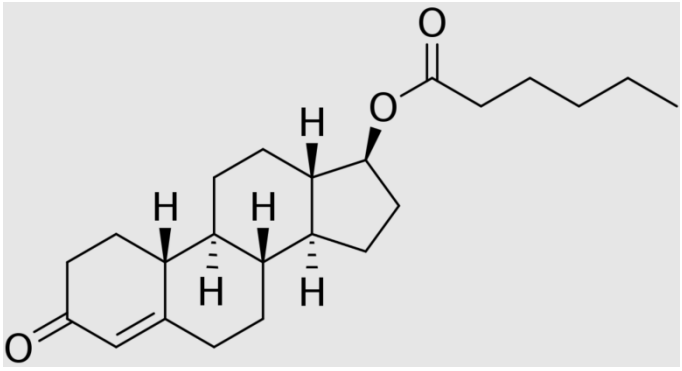


Figure 11: Structure of testosterone caproate [17].

1.8.10 Testosterone Hexahydrobenzoate

Testosterone hexahydrobenzoate which is shown in Figure 12 is an ester of testosterone which is chemically synthesized in the lab. The chemical symbol is $C_{30}H_{48}O_3$. The half-life is 2–4 hours. It is used used to regulate sperm and red blood cell production, muscle strength, fat distribution, bone mass, and sex drive. It can also cause Improves strength of

muscles in the body, sex drive, and bone density. Testosterone hexahydrobenzoate deficiency can cause hypogonadism [17].

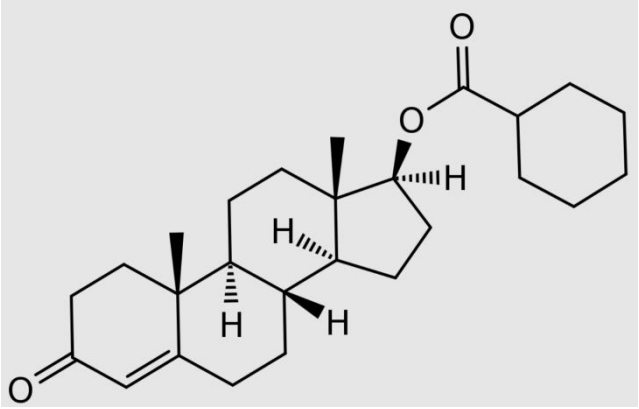


Figure 12: Structure of testosterone hexahydrobenzoate [17].

The testosterone hormone is affected by genomic and non-genomic pathways as shown in the Figure 13; the cytoplasmic androgen receptors bind with the biologically inactive testosterone in an unbound state. Dihydrotestosterone also similarly activates the androgenic receptor (AR). The conformation changes of the receptor are induced by the ligand binders. The testosterone and AR together form a complex structure that functions as a factor for the functional transcription [23]. The transcription initiation is performed by the activated AR receptors. This process of gene expression produces a pool of specific proteins that determine the characteristics of the cells along with their metabolism and activity. In due course, the cascades of the downward signaling process are activated; this produces the genomic effect, which includes activation of the different transcription factors, protein synthesis, and protein activation. When coupled with the G-protein, the reactors lead to the activation of phospholipase C and increase the intracellular Ca^{+}. The

response of the androgen receptor does not include translation or transcription, and it operates much faster against the genomic effects.

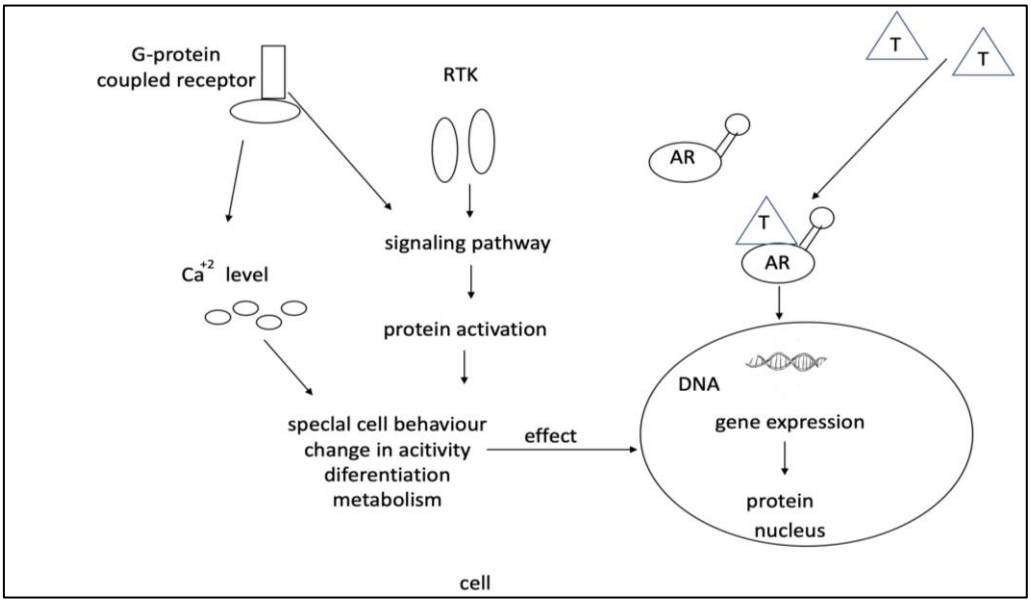


Figure 13: Genomic and non-genomic effects of testosterone.

When activated, the androgen receptor increases the level of Ca^{+}, activates the multiple protein kinases, and facilitates interactions between the proteins; this in turn triggers the important signaling cascades. Cells such as macrophages do not possess the typical androgen receptor; instead, they contain specific sites for binding testosterone on the plasma membrane surface. Such receptors are coupled with the homeostasis of intracellular Ca^{+}. The binding with testosterone stimulates the mobilization of Ca^{+}, which increases its level in the intercellular spaces. The testosterone receptors associated with the membrane are linked with the G-proteins, which are in turn associated with the phospholipase [23]. The androgenic and anabolic impacts of testosterone are known to produce a varied range of morphological traits. Several of these effects require the

conversion of testosterone into a more potent form of androgen, dihydrotestosterone, which plays a key role in the maturing process of the sex organs.

High levels of the endogenous testosterone are known to encourage dominant behaviors in males. This dominant behavior in some cases is manifested as aggressiveness. However, dominance as a characteristic is often not represented by aggressiveness. Nevertheless, in certain cases, aggressive behavior is expressed as a tendency to break norms [23]. Higher levels of endogenous testosterone are directly associated with a higher tendency to take risks and face challenges. Androgens are also known to play the role of modulators for almost all facets of sexual behavior, including autonomic functions such as motivational, emotional, and cognitive functions.

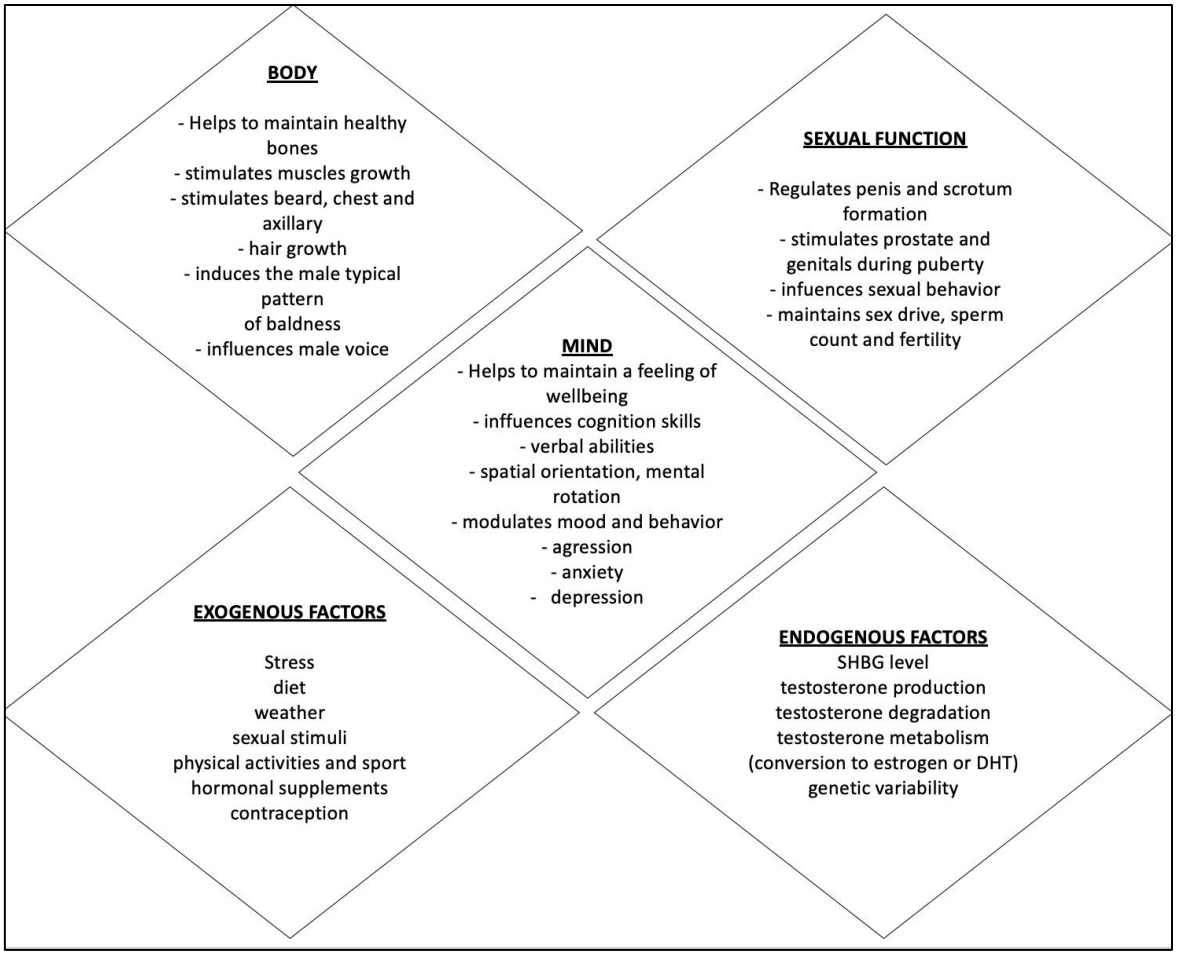


Figure 14: Testosterone activity and factors modulating its effects.

The upwards and downwards arrows in Figure 14 indicate stimulation and attenuation, respectively. Androgen activity is dependent on the level of testosterone, which is in turn affected by various other factors [23]–[26] and this is true in other species as well.

Moreover, testosterone affects the morphology of the body and other characteristics. Mental rotation is key phenomena investigated in the context of gender-specific effects of testosterone.

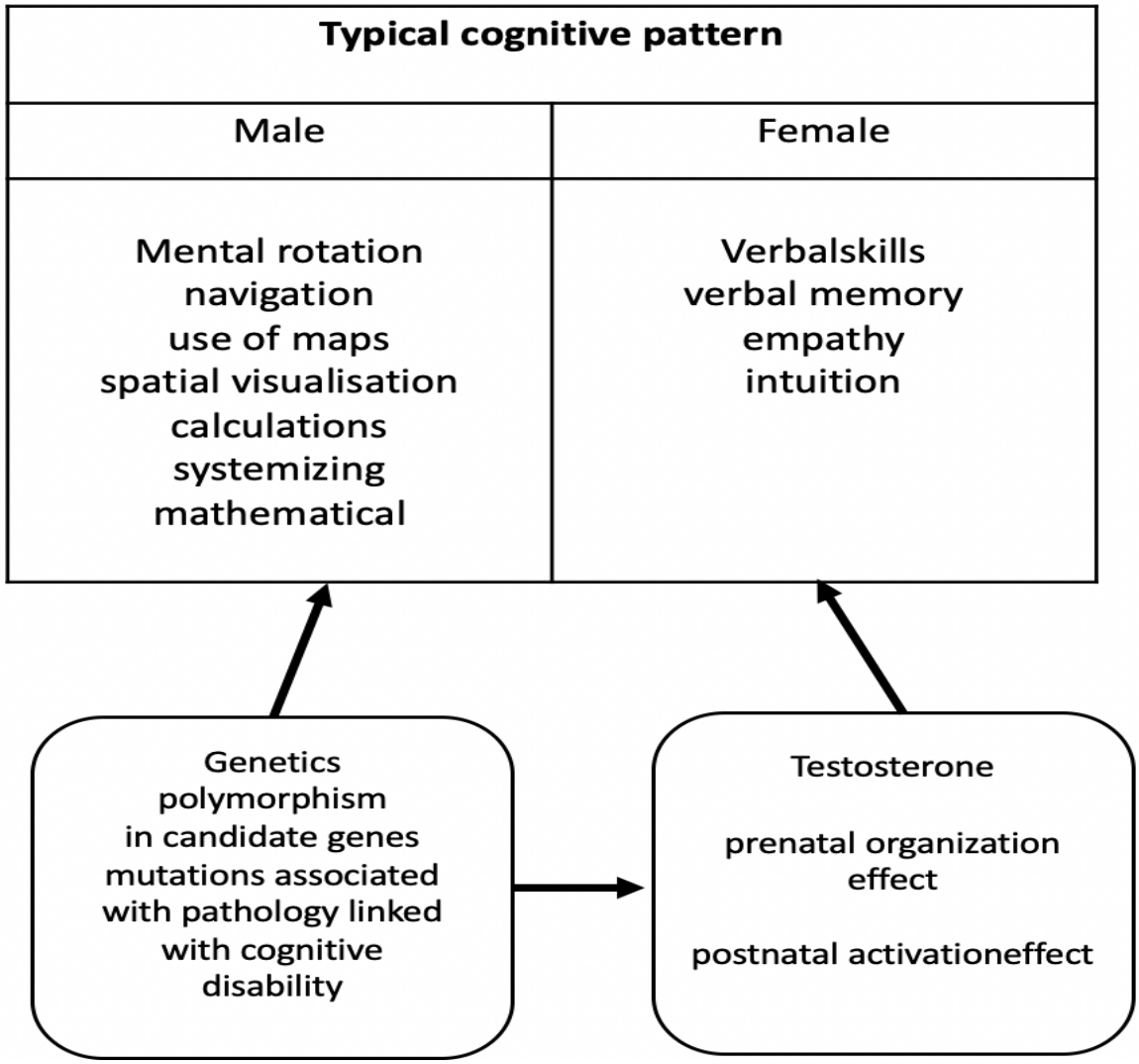


Figure 15: Links between genetic factors, testosterone, and cognitive skills.

Testosterone is known to be associated with spatial rotation [27]. Women with higher levels of testosterone are found to be more active in terms of spatial visualization and mental rotation than those with lower levels. On the other hand, no associations were found between the testosterone levels and spatial abilities of males. Hence, it can be concluded that increased testosterone levels are not associated with amplified male characteristics [28].

Figure 15 depicts the links between testosterone, genetic factors, and cognitive skills. Cognitive performance is determined by different genetic factors. Genetic polymorphism can modulate the final protein product activities. Moreover, polymorphism of the androgen receptor alters its activity as a factor for transcription; androgen insensitivity syndrome is caused by genetic mutations in these receptors. Furthermore, during testosterone metabolism, somatic mutations occur, affecting the biosynthesis of testosterone and the concentration of the bioavailable fraction [23]. The molecular mechanism of testosterone action tends to vary. Dihydrotestosterone and testosterone are ligands of the androgen receptor at the androgen level. The classical genomic pathway of testosterone includes the activation of ligand receptors as a key transcription factor in gene expression, including cell differentiation, proliferation, apoptosis, and metabolism.

The metabolism testosterone from cholesterol proceeds with the help of desmolase activity. It can be seen in Figure 15 that pregnenolone is converted into testosterone through a series of intermediary products: 17-alpha-hydroxypregnenolone, dehydroepiandrosterone, and 4-androstene-3, 17 dione, respectively. An alternative available pathway metabolizes testosterone in the body, including intermediates such as pregnenolone, 17-alpha-hydroxyprogesterone, and progesterone. The testosterone hormone binds to the sex hormone (i.e., SHBG) during circulation [23]. Because of this binding activity, testosterone is protected from degradation during metabolism and becomes biologically inactive. By this stage, only a small fraction of the hormone is in the free and active stage and therefore able to bind to the receptor for further metabolism. In certain tissues (e.g., brain and adipose tissues), the testosterone is converted into the female sex steroid estradiol via the catalyzation of aromatase.

1.9 Studies Conducted to Identify Steroids in Animal Hair

Table 1 gives a comparison of the methods published for analysis of hair in different categories of cattle's and other animals. The table compares the following important details. Detection methods used, type of mobile phase employed, types of steroids detected, column used for chromatographic separation, extraction of steroids from hair discussed along with their respective LOD and LLOQ where possible. Overall, the LCMS/MS and GCMS methods has been more sensitive, reliable and reproduceable. EIA and RIA methods in general suffers from cross reactivity issues along with poor sensitivity in most cases. Methanol and formic acid have been widely used as solvents as mobile phase in LCMS/MS method. Often a reverse phase C18 column has been used for chromatographic separation. Mostly a combination of liquid-liquid extraction is followed by solid phase extraction which makes the extraction process very labour intensive. The extraction method we report here is a lot simpler and less laborious and it is also very robust and reproduceable.

Table 1: Methods used for determining different steroids in the hair of wild species.

Year	Animal	Detection method and mobile phase	Steroid detected and column used	Extraction Method	LOD	LLOQ	Reference
2017	Domestic cattle breeds	QuadrupoleLC–MS/ MS. Mobile phase solvent A consisted of ammonium formate (200 mM) with 0.1% formic acid and eluent B consisted of ammonium formate (200 mM) in methanol.	Cortisol Column: 50 × 2.10 mm 2.6 µm C18 column	20 mg hair snippets were extracted in 3 ml methanol for 16 h overnight in an ultrasonic bath at 55 °C	0.2 pg/mg/0.1 pg/mg	1 pg/mg/0.5 pg/mg	[29]
2015	House rat/mouse	LC–MS/ MS The mobile phase was a mixture of methanol and water (90:10 v/v) containing 2.0 mM ammonium acetate, which was filtered through micro porous membrane (0.22 µm) before use	Cortisol Column:5 µm, 150 × 4.6 mm, Platisiltm ODS-C_{18}	20 mg of hair pieces was incubated in 1 ml of methanol for 24 h. followed by the solid phase extraction using (SPE) C18 column	House rat/House mouse 0.5/1.25 pg/mg	1.25/2.5 Pg/mg	[30]

Table 1: Methods used for determining different steroids in the hair of wild species (Continued).

Year	Animal	Detection method and mobile phase	Steroid detected and column used	Extraction Method	LOD	LLOQ	Reference
2015	Domestic cattle: Holstein	EIA	Cortisol	20 mg was taken 1 ml methanol added and incubated at 100 rpm at 50°C	The sensitivity of the cortisol assay kit was 0.01 µg/dL. LOD:1 pg/mg	2 pg/mg	[31]
2009	Bovine hair	LC–MS/ MS Mobile Phase: solvent A, water–acetonitrile–methanol–formic acid (300:350:350:20, v/v/v/v); solvent B, acetonitrile–methanol–formic acid (500:500:20, v/v/v))	Estradiol benzoate (EB), testosterone cypionate (TC) and testosterone decanoate (TD) Column: Waters acquity UPLC BEH C18 analytical column of 100 × 2.1 mm and 1.7 µm particle size	200 mg Hair was digested with the 2ml reduction agent tris(2-carboxyethyl) phosphine hydrochloride (TCEP) for 1 hour, followed by extraction using SPE C18 cartridges	2 pg/mg for all	5 pg/mg for all	[32]
2009	Himalayan Tahr	RIA	Testosterone	50 mg hair was collected and incubated with 3 ml of 1 M NaOH for 40 min, at 38 °C. A 300 µl aliquote was extracted with 5 ml of diethyl ether	0.9 pg/mg		[33]

Table 1: Methods used for determining different steroids in the hair of wild species (Continued).

Year	Animal	Detection method and mobile phase	Steroid detected and column used	Extraction Method	LOD	LLOQ	Reference
2007	Domestic horse (Equus ferus caballus)	GC–MS/ MS The following temperature ramp was used: 150°C (1 min) to 320°C at 18°C/min. The temperature of the source was 280°C and the transfer line was set to 300°C.	Testosterone Column: silica capillary column DB5MS, 30 m × 0.25 mm i.d. × 0.25 µm	Tail hair samples (100 mg) were dissolved in 1 mL of sodium hydroxide for 15 min at 95 °C. Followed by diethyl ether extraction and a SPE Isolute C18 cartridges eluted with methanol	1 pg/mg	2 pg/mg	[34]
2018	Dogs	LC-MS/MS Mobile phase: Solvents were 0.1%formic acid:0.1% ammonium formate (1:1 V/V)(A) and Methanol: Acetonitrile (25:75)(%)(B)	Testosteron propionate, T. phenyl propionate, T. isocaproate, T. Decanoate Column: Poroshell 120 EC-C18 column (3 mm x 50mm, 2.7 µm particle size)	100 mg hair obtained now 4 mL of 0.1 M phosphate buffer at pH 9.5 was added to the sample. The sample was then ultrasonicated for 60 min, the extracted with 2 x 4 mL of hexane: ethyl acetate (7:3, V/V). This was followed by solid phase extraction (SPE) using Bond Elut NH2 cartridges	0.05 pg/mg	0.1 pg/mg	[35]

Chapter 2: Materials and Methods

2.1 Materials

Testosterone, testosterone isocaproate, testosterone enanthate, testosterone benzoate, testosterone cypionate, testosterone undecanoate, testosterone valerate, testosterone laurate, testosterone caproate, testosterone hexahydrobenzoate, and testosterone undecenoate were all purchased from Sigma Aldrich (Germany); the cortisol-D4 internal standard was supplied by Labco Ltd., UAE. Methanol, hexane, formic acid, deionized water, and LC–MS-grade water and acetonitrile were purchased from Emirates Scientific and Technical Supplies L.L.C. (ESTS) (Dubai, U.A.E.).

An Ascentis Express F5 column (150 mm × 2.1 mm × 2.7 μm), plastic bags, glass test tubes (5 ml), glass Pasteur pipettes. was received from Gomet (Al-Ain, UAE). Millex Samplicity TM Filters and 0.20 μm hydrophilic polytetrafluoroethylene (Merck Millipore, Carrigtwohill, Ireland, cat. no. SAMPLG001), 2 ml glass chromatographic vials with caps (2 ml); were purchased from Emirates Scientific Supplies Ltd., UAE.

2.1.1 Preparation of Standard Solutions

The stock solutions of each testosterone metabolite and internal standard were prepared in methanol with a concentration of 1 mg/ml. All stock solutions were kept and stored in amber vials at -20°C. The working standards solution for the testosterone esters and internal standard were prepared via serial dilution of stock solutions in methanol, to obtain the desired concentrations.

2.1.2 Collection of Hair Samples

The hair samples of 24 camels were collected from a farm in Al-Ain, UAE with length 1.5-5 cm and mass of about a thumb thickness. The hair samples were cut specifically from the hump (i.e., the large lump on the camel where fat is stored). This was performed with particular attention, to avoid any damage to the skin. All hair samples were kept individually in clear, labeled plastic bags at room temperature. The study protocols were approved by the UAEU research ethics committee. Blank hair was selected from one of these 24 camel hair samples. The selection of blank hair war carried out by collecting six different lots of presumed healthy drug-free camels. Samples from male and female camels, with different hair colours (light brown to dark brown) were also included in this evaluation. Selectivity and interference from matrix were determined by the extraction of six lots of blank camel hair samples and running them on the LC-MS/MS method. No Interfering peaks were detected at the retention time of any of the testosterone esters in any of the samples evaluated. One out of six most clean blank sample was selected for preparation of calibration curve and quality controls.

2.1.3 Hair-Sample Extraction Method

The hair samples were washed with methnol/water and then dried down under a gentle stream of N2 then about 100 mg hair was ground to a powder using a Mini-ball mill (Pulverisette 23, Fritsch, Germany) operating at 50 oscillations per second for 15 minutes. Then, 20 mg of ground hair was carefully weighed out into 24 5 ml labeled borosilicate culture tubes; 100 µl of methanol was added to each hair sample. Then, 25 µl of internal standard cortisol-d4 (concentration: 1 ng/ml) was added to all hair samples after grinding

except the blank matrix one, and the samples were incubated in an ultrasonic bath (Branson 5800, Danbury, USA.) at 40°C for 120 min. Samples were incubated in a sonic bath at 50 Hz, 55°C for 240 min before being removed. Next, the samples were vortexed for 1 minute at room temperature, and 1000 µl hexane was added to the mixture for extraction. Samples were vortex-mixed again for 1 minute at room temperature.

Next, the tubes were centrifuged at 1300xg for 20 minutes (Beckman TJ-6, Beckman, UK). After centrifugation, the top organic layer was separated into new glass test tubes using Pasteur pipettes. Then, the extract was aliquoted into tubes and dried under a stream of nitrogen gas at 40°C (Techne, Bibby Scientific, USA). The ground samples were then extracted using 5 µl of high-performance liquid chromatography (HPLC)-grade methanol. Finally, the dry extract was reconstituted with 100 µl of methanol and placed in 2 ml HPLC vials with glass inserts, to be injected into the LC-MS/MS system.

2.1.4 Liquid Chromatography and Mass Spectrometry

The chromatographic separation of testosterone esters was performed using an Agilent 1260 HPLC system on a reversed-phase column [Zorbax Eclipse Plus C18 (Agilent Technology, Santa Clara, CA, USA)] with a particle size of 2.1 µm, an inner diameter of 1.8 mm, and a length of 50 mm. The chromatographic column was maintained at a constant temperature of 35°C and a constant mobile phase flow rate of 0.4 mL/min. The injection volume for analysis into the LC–MS/MS system was 4 µl. The sample contamination was minimized by a wash program that rinsed the needle with a methanol/water (75/25, v/v) mixture. Two mobile phases were used during analysis: Mobile Phase A consisted of 35% acetonitrile and 35% methanol, 30% water, with dissolved 2 ml of formic acid (all LC–

MS grade); meanwhile, Mobile Phase B consisted of 50% water, 50% acetonitrile, with dissolved 2 ml of formic acid (all LC–MS grade).

Optimal testosterone metabolite chromatography conditions were achieved using a binary mobile phase gradient pump, where gradient elution was conducted as follows: Mobile Phase A was held at 100% whilst B was kept at 0.0%; then, this was changed to 100% for B and 0% for A over 30 s. The gradient was held at this ratio for 7.5 minutes. Between 7.5 and 8 min it was brought back to 100% A and 0% B, and the same gradient was then maintained for 4 min. The mass spectrometry analysis was performed using an Agilent 6420 Triple Quadrupole Mass Spectrometry system in the positive electrospray ionization (ESI^+) mode. The electrospray voltage was set at 4000 V, the ion source gas 1 (a desolvation gas consisting of 99.9% nitrogen) pressure was set at 20 psi, the ion source gas 2 (a nebulizer gas consisting of nitrogen) was set at 45 psi, and the drying gas (N_2) flow was 8 L/min at 325°C.

The HPLC system consisted of a pump, auto-sampler, column oven, and degasser controlled by Aglient MassHunter™ software. Moreover, the auto-sampler component had a high injection speed, and the system could use multiple solvents simultaneously. The Tandem Mass Spectrometer system consisted of a triple quadrupole LC-MS/MS system, Model 6420, Agilent Technologies, USA Ltd, with a newly developed UF-Qarray ion guide; this increased the LC-MS/MS sensitivity by enhancing the ion signal intensity and reducing noise, providing a new level of speed and responsiveness. The software employed to control the HPLC parameters (flow rate, mobile phase gradient, injection volume, column temperature, and pump) is shown in Table 2.

Table 2: Factors and conditions used to optimize chromatography for testosterone esters using LC–MS/MS system.

HPLC Parameters	HPLC Conditions
Stationary Phase	Reversed-phase column Zorbax Eclipse Plus C18 (dimensions: 50 mm × 2.1 mm × 1.8 µm)
Mobile Phase A	2 ml formic acid, 350 ml acetonitrile, 350 ml methanol, and 300 ml water
Mobile Phase B	2 ml formic acid, 500 ml water, and 500 ml acetonitrile
Injection Volume (µl)	4.00
Flow Rate (ml/min)	0.40
Column's Temperature (^{0}C)	30.00
Pump mode	Binary gradient
MS Parameters	MS Conditions
Ion Source	Electrospray ionization (ESI)
ESI Mode	Positive
Nebulizing gas lfow (L/min□	2.00
Drying gas flow (L/min)	8.00
Heating gas lfow (L/min□	8.00
Interface temp. (^{0}C)	300.00
Heating block temp. (^{0}C)	400.00

2.1.5 Method Development and Validation

Validation was performed in accordance with the US Food and Drug Administration (FDA) guidance, which suggested that the bioanalytical method should be developed and validated by optimizing the conditions and procedures for the extraction and detection of the analyte and its esters [36]. Moreover, the bioanalytical parameters (i.e., calibration curve, quality control samples, specificity, sensitivity, accuracy, precision, recovery, and linearity) must be involved in the method development and validation, to ensure the suitability of the method for study sample analysis. Three quality controls (QCs), quality control high (QCH), quality control medium (QCM), and quality control low (QCL) were used to determine linearity, intra and inter-day accuracy, and intra- and inter-day precision. The concentrations for each QC level of certain testosterone esters were 500 pg/mg, 125 pg/mg, 31.25 pg/mg and 3.9 pg/mg for QCH, QCM, QCL and QCL-L respectively. Standard addition method was used for analysis of endogenous testosterone, while all other esters were analysed by spiking blank camel hair (known to be free from any exogenous testosterone esters). The selection of blank hair war carried out by collecting six different lots of presumed healthy drug-free camels. Samples from male and female camels, with different hair colours (light brown to dark brown) were also included in this evaluation. Selectivity and interference from matrix were determined by the extraction of six lots of blank camel hair samples and running them on the LC-MS/MS method. No Interfering peaks were detected at the retention time of any of the testosterone esters in any of the samples evaluated. One out of six most clean blank sample was selected for preparation of calibration curve and quality controls.

The extraction efficiency was evaluated by determining the recovery percentage. The recovery was implemented by preparing two sets of quality controls, where each set consisted of three levels of quality control (QCH, QCM, QCL) and each level was prepared six times. In the first set, the quality control samples for QCH, QCM, and QCL were spiked with methanol; then, the internal standard was added to all QC samples; however, the extraction method was not performed; hence, this set contained unextracted QC samples. In the second set, the quality control samples for QCH, QCM, and QCL were spiked with blanks; then, the internal standard was added to all QC samples; next, extraction was performed; as a result, this set included extracted QC samples. The area under the normal peak in the chromatogram for the extracted QC samples was compared with that for unextracted QC samples; then, the recovery percentage was calculated. In all six sets, the quality control samples for QCH, QCM, and QCL were spiked with blank hair. In the first set, the internal standard was added to all QC samples; then, extraction was performed. In the second set, the internal standard was added to all QC samples; then, the extraction was performed using 1 ml hexane. The specificity experiment was executed by analyzing six blank camel-hair samples; then, each sample chromatogram was compared with other samples, to detect interferences (e.g., matrix components and impurities). Samples were vortex-mixed again for 1 min. The specificity experiment was performed by analyzing four blank hair samples; then, chromatogram of each sample was compared with that of other samples, to detect interferences (e.g., matrix components and impurities).

Chapter 3: Results and Discussion

3.1 Chromatogram of Major Testosterone Esters with Their Respective Retention Times and Blank Sample

The chromatographic conditions facilitated the separation, identification, and quantitation of testosterone esters; this was achieved using a reversed-phase column [Zorbax Eclipse Plus C18 (Agilent Technology, USA)] with an inner diameter of 2.1 mm, and a particle size of 1.8 μm, and a length of 50 mm along with an Agilent Eclipse Plus C18 guard cartridge (3 mm × 5 mm × 1.8 μm) (Agilent Technologies, U.S.A.). Using chromatographic separation, this method could effectively separate the major testosterone esters and their metabolites . Although these major testosterone esters and metabolites have similar structures and masses, a difference was observed between them in terms of their retention times, as shown in Table 3. The combination of HPLC and MS/MS components provides a powerful tool for detecting and confirming the presence of drugs in complex biological matrices. This technique offers high specificity and sensitivity, and it is widely applied for the simultaneous identification of testosterone and other steroids.

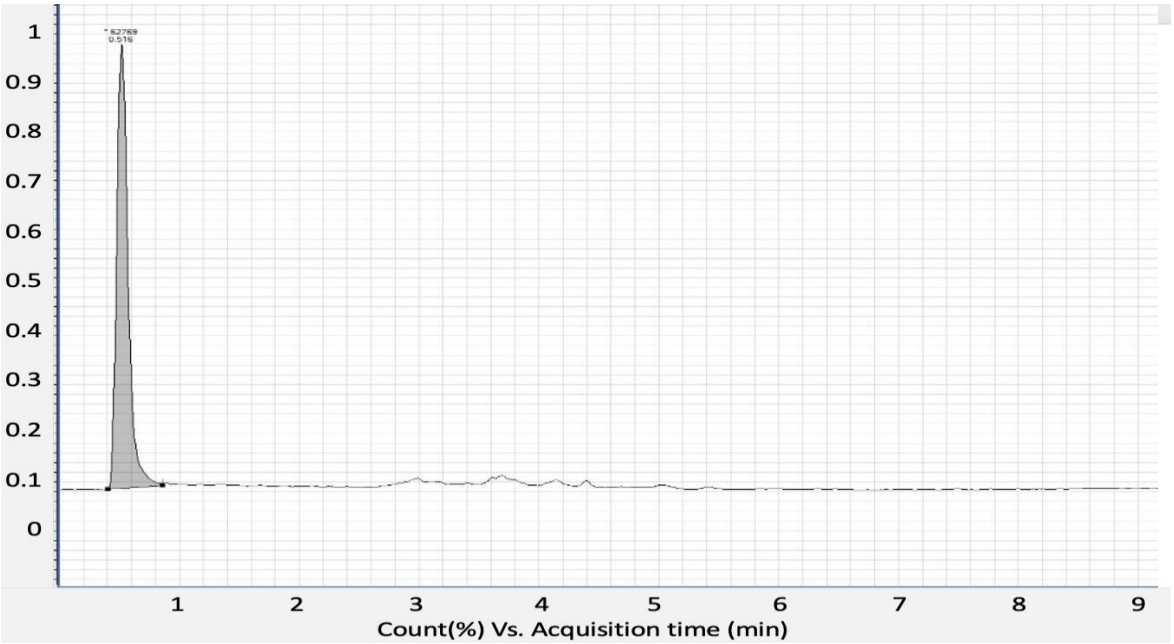


Figure 16: Blank-hair chromatogram demonstrating method specificity.

The most popular versions of the LC–MS method are the tandem mass-spectrometry and "multiple reaction monitoring" (MRM) modes. These approaches use tandem mass-spectrometers to detect a specific product ion generated from a precursor ion (the parent compound) under a given set of fragmentation conditions. The monitoring of the precursor-to-production facilitates the specific and accurate determination of given analytes as shown in the figure 16, even if they are not chromatographically resolved in the liquid chromatography stage of the LC-MS/MS method. Initial experiments were conducted using pure analytical standards to identify the precursor and product ions for specific analytes. Their structures and abbreviations, the mass-to-charge ratios (*m*/z) for the precursor and products ions, and the fragmentor voltage and collision energies values determined for the various analytes are summarized in Table 3.

According to Table 3, although testosterone esters exhibit almost identical retention times and polarities, their masses differ; thus, they are separatable via their mass-to-charge (m/z) ratios. These esters have identical mass-to-charge (m/z) ratios; however, their polarities are slightly different; hence, they can be separated by chromatography, using the aforementioned reversed-phase column as shown in Figure 17.

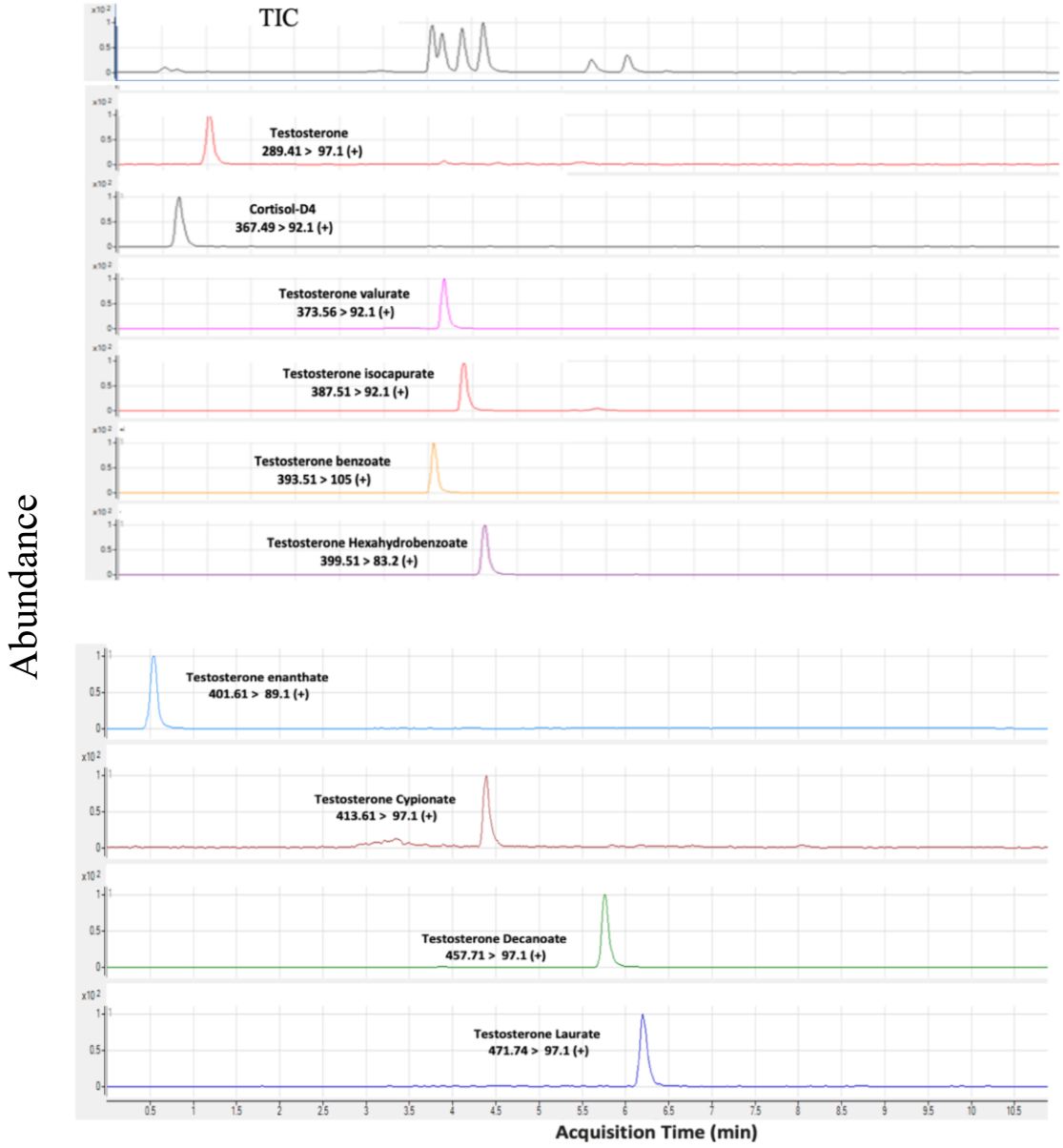


Figure 17: Representative chromatogram of testosterone esters separated by LC–MS/MS.

Table 3: Names, structures, and masses of testosterone esters and their respective retention times

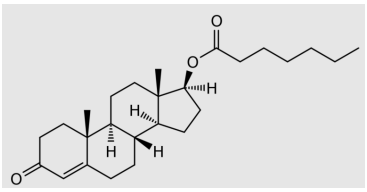
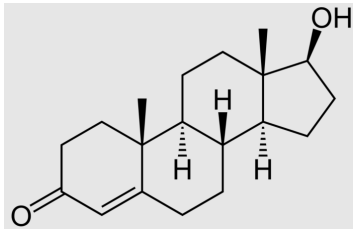
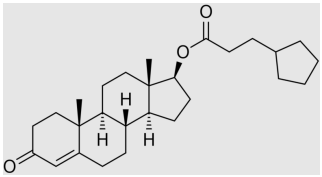
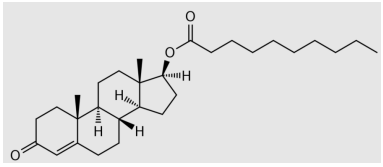
Testosterone and its esters	**Structure**	**Molar Mass (g/mol)**	**[M+H]**	**Retention Time (min)**
Testosterone enanthate (4-Androsten-17β-ol-3-one Enanthate)	 $C_{26}H_{40}O_3$	400.59	401.61	0.52
Testosterone benzoate (4-Androsten-17β-ol-3-one Benzoate)	 $C_{26}H_{32}O_3$	392.53	393.51	3.7
Testosterone cypionate (4-Androsten-17β-ol-3-one Cypionate)	 $C_{27}H_{40}O_3$	412.6	413.61	3.34
Testosterone decanoate (4-Androsten-17β-ol-3-one Decanoate)	 $C_{30}H_{48}O_3$	456.7	457.71	5.659

Table 3: Names, structures, and masses of testosterone esters and their respective retention times (Continued)

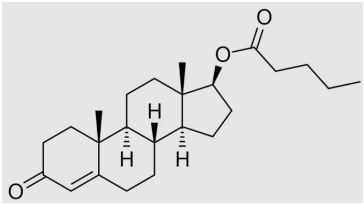
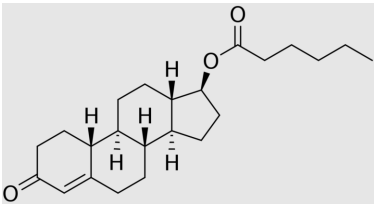
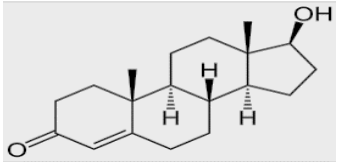
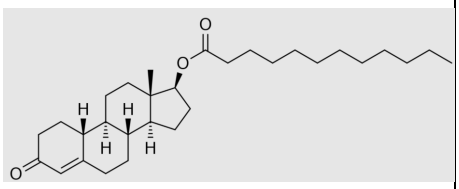
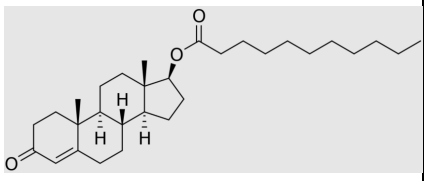
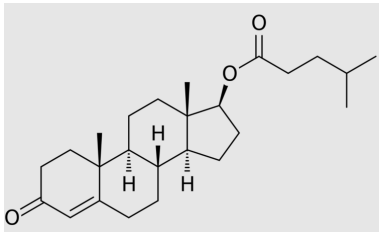
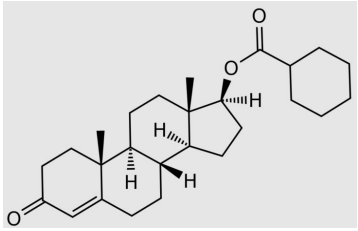
Testosterone and its esters	**Structure**	**Molar Mass (g/mol)**	**[M+H]**	**Retention Time (min)**
Testosterone valerate	 $C_{24}H_{36}O_3$	372.5	373.56	3.827
Testosterone caproate	 $C_{25}H_{38}O_3$	386.6	387.51	3.04
Testosterone (4-Androsten-17β-ol-3-one)	 $C_{19}H_{28}O_2$	288.414	289.44	1.07

Table 3: Names, structures, and masses of testosterone esters and their respective retention times (Continued)

Testosterone and its esters	**Structure**	**Molar Mass (g/mol)**	**[M+H]**	**Retention Time (min)**
Testosterone laurate	 $C_{31}H_{50}O_3$	470.73	471.74	6.59
Testosterone undecanoate (4-Androsten-17β-ol-3-one Undecanoate)	 $C_{30}H_{48}O_3$	456.7	457.71	6.093
Testosterone isocaproate (4-Androsten-17β-ol-3-one Isocaproate)	 $C_{25}H_{38}O_3$	386.58	387.51	4.08
Testosterone hexahydrobenzoate (Testosterone 17β-cyclohexanecarboxylate)	 $C_{26}H_{38}O_3$	398.58	399.59	4.34

Despite the similar molecular weights of several testosterone esters, the chromatographic conditions established in this study facilitated the separation, identification, and quantitation of analytes with different retention times.

3.2 Determination of Method Validation Parameters

The method validation parameters (i.e., intra and inter-day precision, intra- and inter-day accuracy, recovery, linearity, and specificity) were calculated by analyzing six QC samples at three different concentration levels (QCL, QCM, and QCH). The QCs values were used to calculate the percentages of intra and inter-day precision, using the following equation:

$$\% \text{ CV} = (\text{Standard deviation}) \div (\text{mean}) \times 100.$$

The percentages of intra and inter-day accuracy were calculated using the following equation:

$$\% \text{ Accuracy} = (\text{mean value}) \div (\text{nominal value}) \times 100.$$

The extracted and unextracted QCs values were used to calculate the percentage of absolute recovery, using the following equation:

$$\% \text{ Recovery} = (\text{mean extracted QC values}) \div (\text{mean unextracted QC values}) \times 100.$$

The instrument's sensitivity toward testosterone esters was measured using the lower limit of detection (LOD); this was determined by comparing the peak intensities for the lowest analyte concentration against the noise level of the chromatogram. The lowest analyte concentration should exhibit a response three times larger than the background or noise

level. The linear range of several testosterone esters was 0.9–1000 ng/ml, with a very good regression value (linearity), as shown in Table 4. The LOD was 0.48.

The method validation parameters were identified within an acceptable range for each QC level. For example, the percentages of the variation and accuracy coefficients indicate that the method used to analyze testosterone esters offers good intra and inter-day precision and accuracy. Moreover, the method demonstrates a strong recovery percentage, which means that the extraction method is efficient at extracting testosterone esters from hair samples, as shown in Table 4. Table 4 presents the LOD and LOQ values for 24 testosterone esters, as assessed by LC–MS/MS; hence, the method is extremely sensitive. Moreover, the table shows the intra and inter-day precision and accuracy results of six QC samples for each QC level. Furthermore, the values of percentage recovery were calculated by comparing the areas under the normal curve in the chromatogram for the extracted and unextracted QC samples. A low LOD was determined from the lowest concentration of testosterone esters detectable by the instrument. The LOD for this method was 0.48, with a linear range of 0.9–1000 pg/mg.

Table 4: Method validation parameter values for each QC level.

N	Analytes	Conc. (pg/mg)	Intra-day			Inter-day			Recovery (%)
			Precision (% CV)	% Accuracy	SD	Precision (% CV)	% Accuracy	SD	
1	Benzoate	QCH 500	4.4	91.4	20	5.7	96.3	27.6	85.6
		QCM 125	10.3	98.2	12.7	7.9	96.5	9.6	82
		QCL 31.25	7	108.1	2.4	5.8	106.3	1.9	76.7
		QCL-L-3.9	5.5	102.9	0.2	7.9	100.5	0.3	
2	Valerate	QCH 500	2.2	93	10.3	8.2	101.2	41.4	89.4
		QCM 125	11.1	87.6	12.2	8.1	91.6	9.3	96.5
		QCL 31.25	8.5	109	2.9	10.6	102.6	3.4	76
		QCL-L-3.9	3.4	86.5	0.1	12.8	97.7	0.5	
3	Isocaproate	QCH 500	4.8	91.9	22.1	4.2	94	19.9	81.6
		QCM 125	3.8	89.2	4.2	4.1	91.7	4.7	86.9
		QCL 31.25	7.7	109.3	2.6	11.4	117.8	4.2	73.5
		QCL-L-3.9	11.4	104.3	0.5	9.8	103.4	0.4	
4	Hexahydrobenzoate	QCH 500	0.4	88.4	1.8	3.9	96.5	19	88.2
		QCM 125	2.5	89.9	2.8	9.4	97.8	11.5	94.8
		QCL 31.25	4.9	107.8	1.7	18.8	90.8	5.3	79.2
		QCL-L-3.9	3.2	103.9	0.1	2.3	110.2	0.1	
5	Decanoate	QCH 500	2.6	89.6	11.4	1.2	110.8	6.6	88.5
		QCM 125	9.8	98.4	12	4.2	103.8	5.5	82.7
		QCL 31.25	12.1	100.9	3.8	12.8	118.8	4.7	77.2
		QCL-L-3.9	15	94.4	0.6	5	106.2	0.2	

Table 4: Method validation parameter values for each QC level (Continued)

N	Analytes	Conc. (pg/mg)	Intra-day			Inter-day			Recovery (%)
			Precision (% CV)	% Accuracy	SD	Precision (% CV)	% Accuracy	SD	
6	Undecanoate	QCH 500	3.8	98.1	18.8	2.9	85	12.3	88.6
		QCM 125	3.8	103.7	4.9	7.1	103.5	9.2	81.9
		QCL 31.25	4.2	104.8	1.4	6.9	102.7	2.2	78.4
		QCL-L-3.9	3.7	97.7	0.1	4.2	106.9	0.2	
7	Laurate	QCH 500	4.3	89	19.3	2.6	105.2	13.4	90.3
		QCM 125	9.1	96	10.9	10.3	89.8	12.7	81.7
		QCL 31.25	7.4	105	2.4	9.1	95.8	2.7	76.7
		QCL-L-3.9	2	18.1	0.1	3.3	106.9	0.1	
8	Testosterone	QCH 500	1.4	107.4	7.6	1.7	88.8	7.5	88.4
		QCM 125	3.7	108.9	5	2.9	105.6	3.9	84.6
		QCL 31.25	6	93.8	1.8	5.5	105.9	1.8	77.2
		QCL-L-3.9	14	97.5	0.5	4.6	108.7	0.2	

Table 5 shows the analytes names, the mass of all testosterone esters, the precursor and product ions, and the collision energies of all testosterone esters. The precursor and product ions together are referred to as MRM transitions.

3.3 Identification of Precursor and Product Ions

Table 5 shows the types and quantities of testosterone esters found in 24 camel-hair samples. With their masses, precursors, product ions, and collision energies.

Table 5: Analyte names, masses, precursors, product ions, and collision energies

No.	Analytes	Mass (g/mol)	Precursor (m/z)	Product (m/z)	Collision energy (eV)
1	Testosterone	288.4	289.41	97.1 109 79.2 77.1	21 21 61 77
2	Testosterone enanthate	400.6	401.61	392.8 384.4 89.1	5 9 17
3	Testosterone benzoate	392.5	393.51	77.2 105 97.1 109	85 21 25 41
4	Testosterone isocaproate	386.5	387.51	81.2 96.2 97.1 79.2	33 25 29 73
5	Testosterone cypionate	412.6	413.61	79.1 97.1 238	41 25 21
6	Testosterone laurate	470.73	471.74	97.1 109.1 57.3 81.2	29 29 45 61

Table 5: Analyte names, masses, precursors, product ions, and collision energies (Continued)

No.	Analytes	Mass (g/mol)	Precursor (m/z)	Product (m/z)	Collision energy (eV)
7	Testosterone hexahydrobenzoate	398.58	399.59	83.2 231.7	29 93
8	Testosterone valerate	372.55	373.56	97.1 109	29 37
9	Testosterone decanoate	456.70	457.71	97.1 109 95 276.4	37 33 37 13
10	Testosterone capurate	288.43	289.44		
11	Testosterone undecanoate	422.62	443.63	97.1 323.3 252.8	37 25 21

3.4 Camel Sample Results

Figure 18 shows the types and quantities of testosterone esters found in 24 camel-hair samples. Interestingly, testosterone was present in all 24 samples. It exhibited the highest concentrations for all samples (ranging from ~10 pg/mg to 185 pg/mg). This was followed by the hexahydrobenzoate, which was present in six different hair samples in various concentrations (from 12.6 pg/mg to 151 pg/mg).

On the other hand, valerate appeared in three different samples with average concentrations of 10.5, 11.7, and 14.98 pg/mg, respectively (as shown in Figure 18). Meanwhile, laurate analytes were detected in five different samples in varying concentrations: 4.83, 14.1, 14.4, 15.1, and 32.1 pg/mg. Finally, decanoate was only detected in Sample 5, with a concentration of 5 pg/mg.

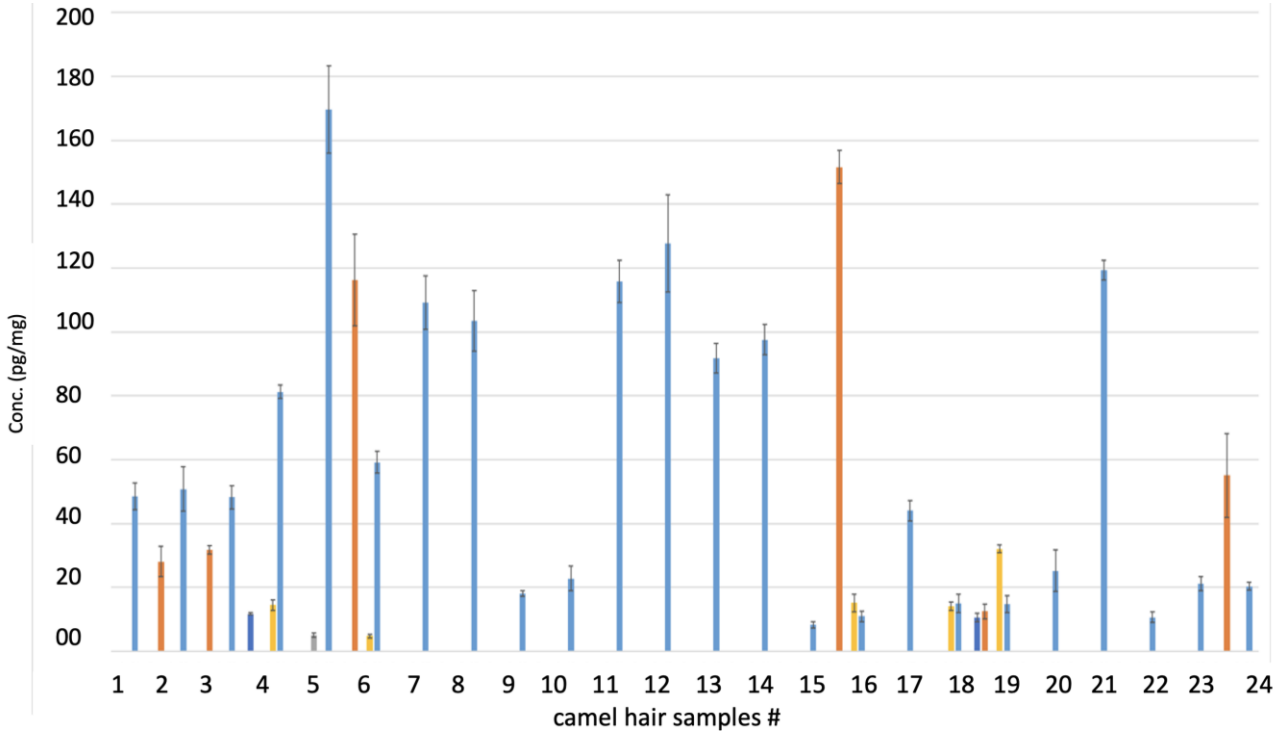


Figure 18:Analysis of the chosen testosterone esters in camel-hair samples, obtained using LC–MS/MS.

Table 6: Comparison between the average concentrations of testosterone esters.

N	Camel-hair samples	Mean ± SD conc. (pg/mg)				
		1	2	3	4	5
		Valerate	Hexahydrobenzoate	Decanoate	Laurate	Testosterone
1	Sample-01	14.9 ± 0.63				48.5 ± 5.95
2	Sample-02		28.1 ± 6.72			50.8 ± 9.95
3	Sample-03		31.8 ± 1.99			48.2 ± 5.11
4	Sample-04	11.7 ± 0.50			14.5 ± 2.34	81.2 ± 2.93
5	Sample-05			5.1 ± 0.85		169 ± 19.24
6	Sample-06		116 ± 20.2		4.8 ± 0.76	59.2 ± 4.89
7	Sample-07					109 ± 11.84
8	Sample-08					103 ± 13.39
9	Sample-09					18.2 ± 1.28
10	Sample-10					22.8 ± 5.59
11	Sample-11					115 ± 9.36
12	Sample-12					127 ± 21.52
13	Sample-13					91.8 ± 6.53
14	Sample-14					97.6 ± 6.82
15	Sample-15					8.35 ± 1.49
16	Sample-16		151 ± 7.35		15.2 ± 3.84	11.1 ± 2.33
17	Sample-17					44.1 ± 4.55
18	Sample-18				14.13 ± 1.82	15.1 ± 4.02
19	Sample-19	10.5 ± 1.81	12.6 ± 3.24		32.1 ± 1.68	14.7 ± 3.66

Table 6: Comparison between the average concentrations of testosterone esters (Continued)

N	Camel-hair samples	Mean ± SD conc. (pg/mg)				
		1	2	3	4	5
20	Sample-20					25.2 ± 9.19
21	Sample-21					119.3 ± 4.31
22	Sample-22					10.7 ± 2.34
23	Sample-23					21.1 ± 3.12
24	Sample-24		55.1 ± 18.5			20.4 ± 1.59

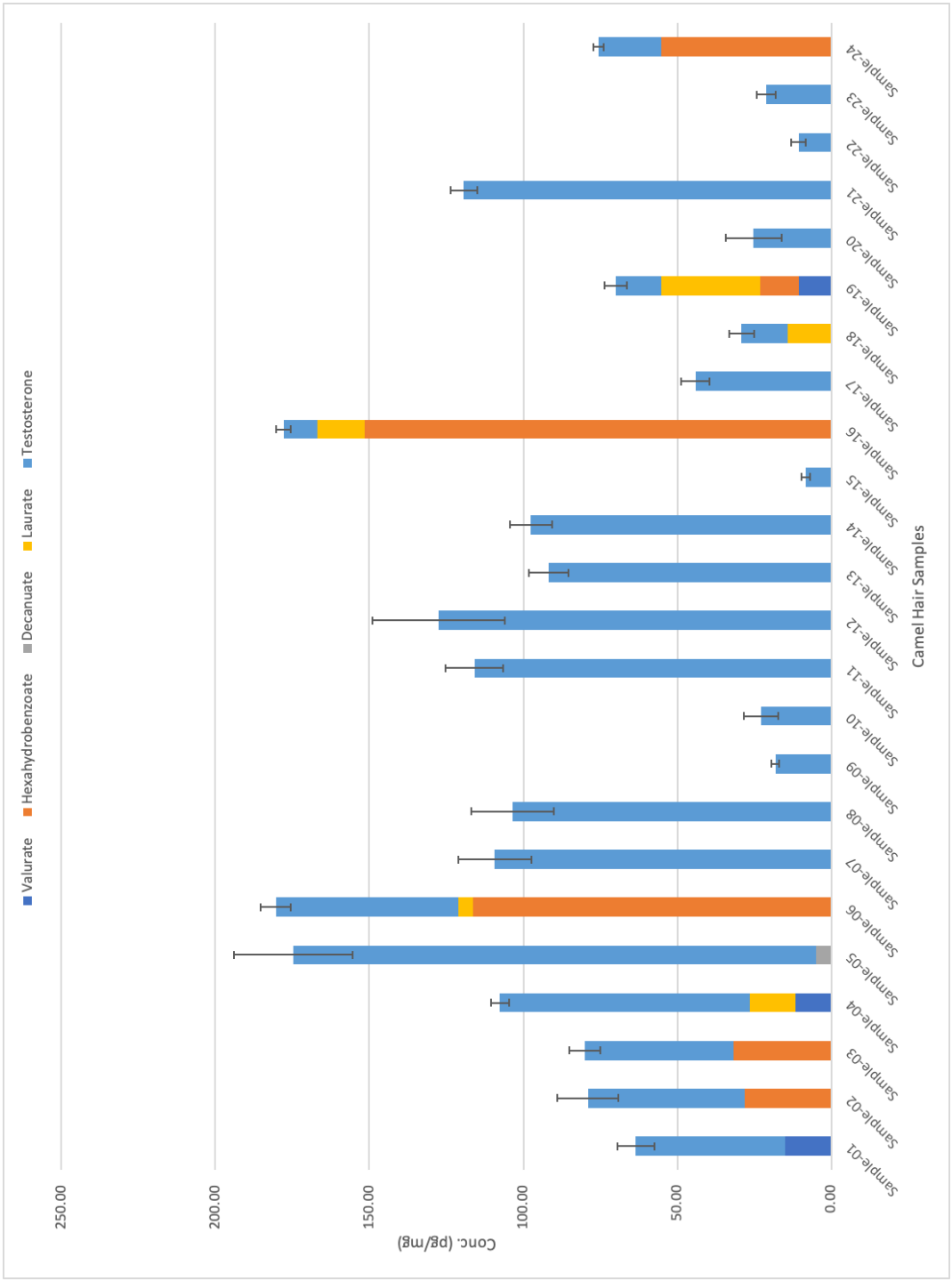


Figure 19: Comparison between testosterone esters concentrations in 24 camel-hair samples. The standard errors of the mean are represented by the error bars in the graph.

Testosterone, its ten esters, and cortisol-D4 internal standard were optimized for LC–MS/MS analysis as shown in figure 19. This was done by running the working solutions by selecting the best precursor and product ions in multiple reaction monitoring (MRM) mode using the Agilent LC-MS/MS optimiser automatic software application. Nothing was administered to camels. However, only eight esters—namely, testosterone, benzoate, valerate, isocaproate, hexahydrobenzoate, decanoate, undecanoate, and laurate—could be verified in camel hair. Only five esters of testosterone could be determined in camel-hair samples, with concentrations of 10.5–14.9 pg/mg for valerate (in three camels), 12.5–151.6 pg/mg for hexahydrobenzoate (in six camels), 4.8– 32.1 pg/mg for laurate (in five camels), decanoate 5.1 pg/mg (in one camel), and 8.35–169 pg/mg for testosterone (all 24 camels). Interestingly, the three racing camels displayed high concentrations of testosterone (59.2–169 pg/mg, all three camels), laurate (4.8–14.5 pg/mg, two camels), hexahydrobenzoate (116 pg/mg, one camel), decanoate (5.1 pg/mg, one camel) and valerate (11.7 pg/mg, one camel). Camels number 3, 4, and 5 were racing camels. And the reason is to increase their performance beyond their natural abilities as well as for the treatment of injuries.

Chapter 4: Conclusion

Doping is a serious threat to the health of animals, and it can have dire health consequences upon the person or animal exposed to it. In this study, an effective method was developed for the determination of testosterone metabolite in camel hair; it is highly sensitive, robust, and reproducible. For extraction, hexane—a low-toxicity solvent—was used. The new method shows high sensitivity and high reproducibility. By using the reversed-phase column Zorbax Eclipse Plus C18, all testosterone esters could be separated. From the linearity results, the quantification method was found suitable for the analysis of camel-hair samples. The author believes that this innovative camel hair test could be used to complement blood, saliva, and urine tests for the detection of testosterone esters in camels. The new hair test could be used to evaluate long-time testosterone doping for injury recovery or performance enhancement. The testosterone hair test for camels could be further extended to cover segmental analysis of camel hair, to detect long-term exposure.

The bio-analytical method was fully validated according to the US Food and Drug Administration (US-FDA) guidelines for the optimization of procedures and conditions, to extract and detect esters. Moreover, the parameters of the bio-analytical method (e.g., its accuracy, linearity, specificity, precision, and recovery) ensure the suitability of this method.

This novel camel-hair test is accurate, sensitive, rapid, and robust. The findings reported in this study could be significant for evaluating racing camels for suspected doping offenses, as well as for injury and disease control and long-term exposure. Further controlled testosterone supplementation studies are necessary to evaluate individual

testosterone metabolite effects on camel health, diseases, and performance enhancement. This new hair test could facilitate further studies in doping control, toxicological, pharmacology, and other clinical fields.

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List of Publications

S. S. Ashraf, W. El-Gahany, A. Alraeesi, L. Al-Hajj, A. Al-Maidalli, and I. Shah, "Analysis of illicit glucocorticoid levels in camel hair using competitive ELISA – Comparison with LC–MS/MS," Drug Test. Anal., vol. 12, no. 4, pp. 458–464, 2020, doi: 10.1002/dta.2750.

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Doping is an acute problem in today's animal racing world, particularly in camel racing. The aim of this study was to develop and validate a liquid chromatographic-mass spectrometric (LC-MS/MS) method to determine testosterone esters in camel hair, and to apply the validated method to determine testosterone esters in collected samples

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