QUANTIFICATION OF ENDOGENOUS ANABOLIC ANDROGENIC STEROIDS USING GAS CHROMATOGRAPHY TANDEM MASS SPECTROMETRY; AN APPLICATION OF STEROID PROFILE FROM *EURYCOMA LONGIFOLIA* ADMINISTRATION

By

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Thesis submitted in fulfillment of the requirements

For the degree of

Master of Science

2011

ACKNOWLEDGMENTS

Foremost, Praise is due to the Almighty ALLAH, the compassionate and most merciful for allowing me to finalise the current Master project safely and hope successfully.

I would like to express my sincere gratitude to my supervisor Prof. Aishah A.Latiff for her unfailing support to my master in its theoretical and practical stages; her patience, motivation, enthusiasm, and for her enriching and valuable comments and suggestions. I extended my thanks and appreciation to Dr. Alsayrafi the General Manager of Anti-doping Lab Qatar for his support.

I am also grateful to Hj. Normaliza for her co-supervision, guidance, and technical assistance throughout my study. Special thanks are due to Dr. Micheal Khalil for his consultation and valuable lectures. Besides, I would like to thank my friend Khadija and fellow friends in Anti-doping lab Qatar for their help and encouragement. I am also indebted to the Polis Tentera Diraja Malaysia for providing 27 volunteers to be involved in this study.

Moreover, my heartfelt thanks go to Qatar Olympic Committee and to Anti-Doping Lab Qatar for granting me a scholarship and for providing the necessary financial support to finalise this work and put it in its final form.

Last but not least, I am very grateful to my brother Salem for his wonderful companionship along the whole period of my study. Sincere thanks are also to my parents, to my dear daughter Noora, my aunt Damtha and all my family members for their great patience and support during this project.

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LIST OF ABBREVIATIONS

CE	collosion energy
DHEA	dehydroepiandrosterone
E	Epitestosterone
E.coli	*Escherichia Coli*
EAAS	Endogenous androgenic anabolic steroids
GC/MS	Gas chromatography mass spectrometry
GC-MS/MS	Gas chromatography tandem mass spectrometry
H^+	Hydrogen ion
HPLC	High performance liquid chromatography spectrometry
LC-MS-MS	Liquid chromatography mass spectrometry
IOC	International Olympic Committee
ISL	International Standard for Laboratories
K^+	Potassium ion
LLE	Liquid-liquid extraction
LOD	limit of detection
LOQ	Limit of quantification
m/z	mass-to-charge ratio
MRM	Multiple Reaction Monitoring
MSTFA	N-methyl-N-(trimethylsilyl) trifluoroacetamide
Na^+	Sodium ion
NATA	National Association of testing Authorities, Australia
^oC	Celsius temperatures
r^2	correlation coefficients
RIA	radioimmunoassay
SIM	selected ion monitoring mode
SPE	solid phase extraction

T	testosterone
T/E ratio	Testosterone to Epitestoerone
TMS	trimethylsilyl
UHQ	ultra-high quality
WADA	World Anti-doping Agency

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ABSTRAK

Kaedah yang menggunakan kromatografi gas-spektrometri tandem jisim (GC-MS/MS) telah dibangun untuk menentukan paras steroid endogenous androgenik anabolik (EAAS) termasuk testosteron, dehidroepiandrosteron, 4-androstenedione, 5-androstenediol, dihidrotestosteron serta beberapa metabolit berkaitan seperti androsteron, etiokolanolon, 5-α-androstandiol dan 5-β-androstandiol dalam sampel urin.Sampel urin telah diekstrak melalui kaedah pengekstrakan Fasa Pepejal (SPE) dan dianalisis melalui teknik GC-MS/MS. Pemonitoran Tindakbalas Multipel (Multiple Reaction Monitoring, MRM) telah diguna untuk analisis tersebut dengan mengguna kan dua transisi MRM sebagai ion kualifikasi dan satu transisi MRM sebagai ion kuantifikasi bagi setiap steroid. Kelinearan, peratus perolehan semula pengekstrakan, LOD, ketepatan dan kejituan analisis intra-hari dan inter-hari dan juga kebolehtahanan kaedah telah dijalankan kajian pengesahan. Korelasi ko-efisikasi (r^2) yang diperolehi adalah melebihi 0.993 untuk semua sebatian. Peratus perolehan semula yang dicapai dengan menggunakan kaedah ini adalah melebihi 70% untuk kesemua analit. Had pengesanan yang diperolehi menggunakan kaedah yang dibentangkan di dalam kajian ini adalah dari julat 0.1 hingga 0.2 ng/mL. Had penentuan pula adalah dari 0.5 hingga 1 ng/mL. Keputusan kajian menunjukkan kejituan yang baik dimana CV < 8% dan ketepatan ≥ 80% untuk kesemua sebatian dan untuk semua peringkat assay. Keputusan pengesahan menunjukkan bahawa kaedah analisis yang digunakan dalam kajian ini

untuk kuantifikasi steroid anabolik dalam urin dapat memenuhi kriteria bagi validasi kaedah kuantitatif mengikut WADA ISL dan Nota Teknikal NATA.

EAASialah suatu kumpulan sebatian semulajadi yang menyerupai testosteron secara kimia dan mempunyai tindak balas androgenik anabolik yang serupa. Administrasi bahan-bahan ini untuk peningkatan prestasi atlit dilarang dalam sukan. Penyalahgunaan steroid dianggap sebagai suatu langgaran yang amat serius dalam sukan. *Eurycoma longifolia* ialah suatu tumbuhan herba yang berasal dari negara Tenggara Asia yang diakui boleh meningkatkan paras testosteron. Justeru itu, ia diguna dalam kalangan atlit dan ahli bina badan untuk merangsang jisim otot, kekuatan atlit dan keseluruhanprestasi. Dalam kajian ini, 27 sukarelawan telah dikenalpasti untuk mengambil *Eurycoma longifolia* untuk jangkamasa 15 hari dan sampel urindari setiap subjek telah dikumpul setiap lima hari. Kuantifikasi EAAS selepas administrasi *Eurycoma longifolia* telah dianalisis dengan menggunakan kaedah GC/MS/MS yang telah dibangunkan. Keputusan dari kajian ini menunjukkan suatu peningkatan signifikan dalam paras testosteron serta prekursor dan metabolit steroid untuk jangkamasa 5 hari. Sungguhpun tiada terdapat perbezaan yang signifikan dalam paras EAAS antara kumpulan atlit dengan bukan-atlit dan juga antara kumpulan yang mengambil *Eurycoma longifolia* dngan kumpulan plasebo, jelas terdapat perbezaan yang signifikan dalam paras steroid mengikut fungsi masa.

Quantification of Endogenous Anabolic Androgenic Steroids using Gas Chromatography Tandem Mass Spectrometry; an application of steroid profile from *Eurycoma longifolia* administration

ABSTRACT

A method was developed using gas chromatography tandem mass spectrometry (GC/MS/MS) to quantify the levels of endogenous androgenic anabolic steroids (EAAS) including testosterone, dehydroepiandrosterone, 4-andro - stenedione, 5-androstenediol, dihydrotestosterone and other related metabolites like androsterone, etiocholanolone, 5-α-androstandiol and 5-β-androstandiol in urine samples. The steroids were extracted using SPE method followed by LLE and analyzed by GC/MS/MS. Multiple Reaction Monitoring (MRM) was adopted for the analysis, using two MRM transitions as the qualifier ions and one MRM transition as the quantifier ion for each analyte. The linearity, extraction recovery, LOD, intra- and inter–day precision and accuracy as well as robustness of the method were validated. The correlation coefficients (r^2) obtained was higher than or equal to 0.993 for all the compounds. The recoveries achieved using the method were higher than 70% for all analytes The limits of detection that was obtained using the method described in the present study ranged from 0.1 to 0.2 ng/mL. The limits of quantification ranged from to 0.5 to 1 ng/mL. The results showed good precision; where CV < 8% and accuracy ≥ 80% for all compounds in all assayed levels. The validation results showed that the analytical method used in this study to quantify the investigated anabolic steroids in urine has complied with the criteria of the validation for quantitative methods according to WADA ISL and NATA Technical Note.

EAAS is a group of natural compounds that are chemically similar to testosterone and share the same androgenic anabolic action. Administration of these substances for enhancement of athletic performance is forbidden in sport. Abuse of these steroids is one of the most serious violations in sport. *Eurycoma longifolia* is an herbal plant native to South East Asian countries which is claimed to boost the testosterone levels. Accordingly, it is used among the athletes and bodybuilders to enhance the muscle mass, strength, and the overall performance. In this study, 27 volunteers were recruited to consume *Eurycoma longifolia* over a period of 15 days and the urine sample of each subject was collected every five days. Quantification of EAAS after administration of *Eurycoma longifolia* was carried out using GC-MS/MS method. Results of the administration study showed a significant increase during the first five days in the urinary levels of the testosterone and related steroid precursors and metabolites. Although there were no significant differences in the levels of EAAS between the athletes and non-athletes group as well as between treated and placebo group, there were clearly significant differences in the levels as a function of time.

CHAPTER 1

INTRODUCTION

Enhancing athletic performance by administrating foreign substances is forbidden in human sports. The global fight against doping in sports is supervised by the World Anti-Doping Agency (WADA). The latter maintains the World Anti-Doping Code, which in return includes the WADA Prohibited List, which defines the substances and methods prohibited in sports. In practice, drug abuse is controlled via testing athletes. Urine or blood samples are collected from athletes, either prior to or during competition. Test samples are analysed in analytical laboratories accredited by WADA to check whether or not they have banned substances.

Anabolic Androgenic Steroids (AAS) are a group of natural and synthetic compounds that are chemically similar to and mimic the actions of endogenous testosterone. In addition to their medical use, AAS has been misused for the past 50 years by a wide variety of athletes in the hope of improving their training, endurance and performance. The use of AAS in sports has been banned since the mid-70s, and it is still the most misused class of drugs in sports today. Steroid abuse has also become more and more prevalent outside the field of sports.

Detecting AAS is considered a challenge due to the presence of numerous different synthetic steroids, their extensive metabolism in the body and to their low concentration in urine. A capillary gas chromatograph coupled to a bench top quadrupole mass spectrometer (GC/MS) is considered the backbone when testing AAS during the last decade. Although the set-up fairly allows successful large scale screening, more efficient instrumental techniques, such as high resolution mass

spectrometry (HRMS), tandem mass spectrometry (MS/MS) and liquid chromatography mass spectrometry (LC/MS) are needed to enhance the selectivity and sensitivity of the measurements. Most of the LC/MS-based methods that are used for the purpose of detecting anabolic steroids use tandem mass spectrometry instruments in a selected reaction monitoring (SRM) mode due to its specificity and selectivity. Additionally, the selection of a specific SRM transition can minimize the endogenous urinary interferences. Using this approach, free or conjugated steroids could be detected in different various matrices, such as human and horse urine, dietary supplements, plasma or hair. The main limitation of this approach (common to all LC/MS approaches) is the poor ionization efficiency of steroids in electrospray ionization; especially, in case of steroids that do not have a conjugated keto moiety (Pozo et al., 2011). In addition, LC typically does not provide as high a resolution as that of the GC and therefore it may pose some difficulty in separating analytes with very closely related structures (Stanczyk & Clarke, 2010).

While the advent of LC/MS/MS in the past decades has resulted in dramatic improvements with respect to sensitivity, specificity and automated measurements of serum steroid hormone, there are still situations where the GC/MS or GC/MS/MS assay provides higher chromatographic resolution and even sensitivity. A particular strength of GC/MS and GC/MS/MS stems from their high applicability to measure large numbers of structurally similar analytes. The recent availability of GC/MS/MS systems extends the capability of drug doping laboratories by providing the ability to screen, confirm, and to quantify prohibited compounds in one run, or even in complex matrices (Latiff & Churley, 2009).

Eurycoma longifolia Jack is a herbal plant that is native to South East Asian countries, mainly to Malaysia and Indonesia. It is popularly recognized as ‘Tongkat

Ali', which means the 'walking stick'; it is derived from the presence of long twisted roots (Bhat & Karim, 2010). It is claimed that the regular intake of root extracts is believed to enhance the testosterone levels. An increased interest has also been created among athletes and individuals, who are involved in body building with respect to using such an extract in enhancing muscle mass, strengthening the overall performance (Bhat & Karim, 2010).

Due to these reasons, this study was proposed to monitor the level of testosterone and other endogenous steroid precursors and metabolites to ensure that the intake of *Eurycoma longifolia* is not categorized as doping. If *Eurycoma longifolia* has the ability to increase the testosterone level, the ratio of testosterone to epitestosterone (T/E ratio) should be closely monitored. Otherwise, the use may result in preventing athletes from practicing any sports event and in prohibiting *Eurycoma longifolia* as a supplement.

In order to establish the effects of *Eurycoma longifolia* on steroids and steroid metabolism, a group of volunteers will be identified to consume *Eurycoma longifolia* over a period of 15 days. Then, the urine of each subject will be collected every five days following the process of administration. Gas chromatography with tandem mass spectrometry techniques will be used to quantify all endogenous steroids that are considered an important indicator to steroid abuse and as important parameters to Athletes' Biological Passport.

The findings of this study will resolve the question of whether *Eurycoma longifolia* can be used among the athletes. If *Eurycoma longifolia* is shown to have a significant effect on the testosterone and other endogenous steroids levels, the National Sport Institute must prohibit athletes from taking *Eurycoma longifolia* as

supplements. Currently, *Eurycoma longifolia* has been widely used as a supplement to increase energy and to enhance the performance of athletes.

1.1 Objectives

There are two main objectives within the current study; that include the following:

1- Developing, optimising and validating a method for quantifying AAS in urine using gas chromatography tandem mass spectrometry using a GC-triple quadrupole system.

2- Quantifying testosterone and other endogenous anabolic androgenic steroids (EAAS) following oral administration of *Eurycoma longifolia* in volunteers' urine.

CHAPTER 2

LITERATURE REVIEW

This chapter is an introductory chapter that presents a general background on the endogenous anabolic androgenic steroids, the method of analysis and the technique used, and a revision reviews on *Eurycoma longifolia* Jack.

2.1 Endogenous Anabolic Androgenic Steroids and Abuse in sport

Abuse of endogenous anabolic androgenic steroids (EAAS) is one of the most serious violations in sport. Such a type of steroids is misused by athletes to increase muscle mass and to enhance the physical performance. The misuse of these steroids in sport has led to issue a ban by the International Olympic Committee (IOC) in 1974 and by the World Anti-doping Agency (WADA) since 2003. The latter organization encouraged sport drug testing laboratories to develop the screening methods that help to detect the anabolic steroids in urine (Fragkaki, 2009). Doping Control Laboratories have been confronted with the challenge of finding criteria that allow endogenous steroids to be distinguished from their synthetic analogues in the urine of athletes.

These EAAS are derived from the naturally occurring steroid testosterone (T), which is considered the most important androgenic steroid (Renterghem, 2008). In spite of the increasing popularity of synthetic steroids, naturally occurring steroids are still widely misused in sports. Intake of endogenous steroids affects altering one or more parameters of the urinary steroid profile which currently include testosterone

(T), epitestosterone(E) , androsterone, etiocholanolone, 5-α-androstandiol and 5-β androstandiol (WADA, TD2009EAAS)

2.1.1 Chemical structure of steroids and Steroidogenesis

Steroid hormones are fat soluble hormones that are derived in specific tissues in the body. As far as the structure of steroids; they have a core frame of four carbon rings, three cyclohexanes, one cyclopentane and additional functional groups that are attached to the rings (Figure 2.1).

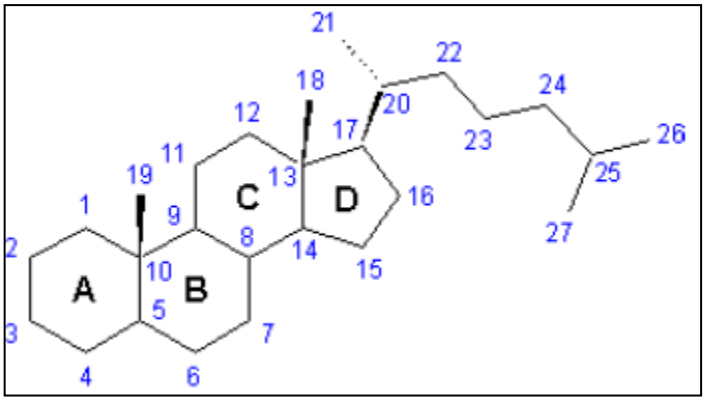

Figure 2.1 General Structure and carbon numbering of steroids

Cholesterol, which contains 27 carbon atoms (C27), is a commonly-known steroid that acts as a precursor to all five major groups of steroid hormones: progestagens (C21), mineralocorticoids (C21), glucocorticoids (C21), androgens (C19), and estrogens (C18). As for steroid hormones, they contain 21 or fewer carbons.

Pregnenolone, a precursor of all other steroid hormones, is the first steroidhormone that is synthesized from cholesterol by the cleavage of a bond between C-20 and C-22. It is further one of the progestagens that maintains menstrual cycles and pregnancy. Pregnenolone is converted into progesterone, a

major precursor of four other steroid hormone groups. Cortisol, a major component of glucocorticoids, is produced from progesterone, which breaks down fat and protein to form glycogen to maintain the normal levels of glucose in blood. Aldosterone, the major mineralocorticoid, is synthesized from progesterone through corticosterone. Mineralocorticoids are responsible for increasing the reabsorption of Na^+, and the excretion of K^+ and H^+. The latter helps increase the amount of water to adjust blood volume and pressure. Progesterone can be converted into androstenedione when a few intermediates go through the cleavage of the two carbon chains at the C-17 position; a process that results in C19 androgens. Testosterone is synthesized from androstenedione by reduction of the keto group on C-17. Androstenedione is also a precursor of estrogens; the predominant one of the three estrogens is estradiol, which is responsible for the development of female secondary sex characteristics (Makin et al., 1995). Estrogens are synthesized by the loss of methyl group on C-19; a matter that results in having C18 structure (Figure 2.2).

2.1.2 Metabolism of Steroids

Steroids are synthesised from cholesterol and then transformed into other steroids. The pathways of steroidogenesis differ among different species; the pathways of human steroidogenesis are shown in the Figure 2.2. The anabolic androgenic steroids are extensively and notably metabolized in the liver and their target tissues. The metabolism of anabolic androgenic steroids generally follows the metabolic pathways observed for testosterone metabolism that has been comprehensively reviewed (Schänzer, 1996 and Van Eenoo & Delbeke, 2006). Metabolism conversion is categorized into Phase I and II reactions. In phase I reactions enzymatically catalyzed alterations are observed, mainly oxidations and

reductions, which help convert the steroids into more polar compounds in order to inactivate the drug and facilitate its elimination from the body. Phase II reactions conjugate the steroids or their metabolites mainly with glucuronic acid or sulphate. They also help in the elimination of steroids from the body (Schänzer, 1996).

Testosterone belongs to the androgen group, which is required for the development of male secondary sex characteristics. Androgens on the other hand embed the following derivatives: dehydroepiandrosterone (DHEA), androstenedione, androstenediol, testosterone (T), androsterone, and dihydrotestosterone. DHEA (Figure 2.3-1) is one of androgen derivatives from pregnenolone without the conversion of progesterone; it represents a different synthetic pathway from testosterone. It has the same molecular weight as testosterone, and shares a similar structure to testosterone among the androgens. Epitestosterone (E), an epimer of testosterone at C-17, is an inactive form of testosterone; it has no anabolic or androgenic effects. Its role in the body and the exact pathway of its formation is still unknown (Bellemare et al., 2005). A study has shown that half of epitestosterone is produced in the testis, and it accumulates in mammary cyst fluid and in the prostate. Antiandrogenic activity and neuro protective effects are also found. Though it varies among individuals, the ratio of epitestosterone to testosterone that is naturally excreted into human urine is approximately 1:1 (Bellemare et al., 2005). Glucuronide conjugates of testosterone and epitestosterone are formed by glucuronosyltransferase in liver microsomes and uridine diphosphoglucuronicacid, and excreted in urine through steroid hormone metabolism (Schänzer, 1996).

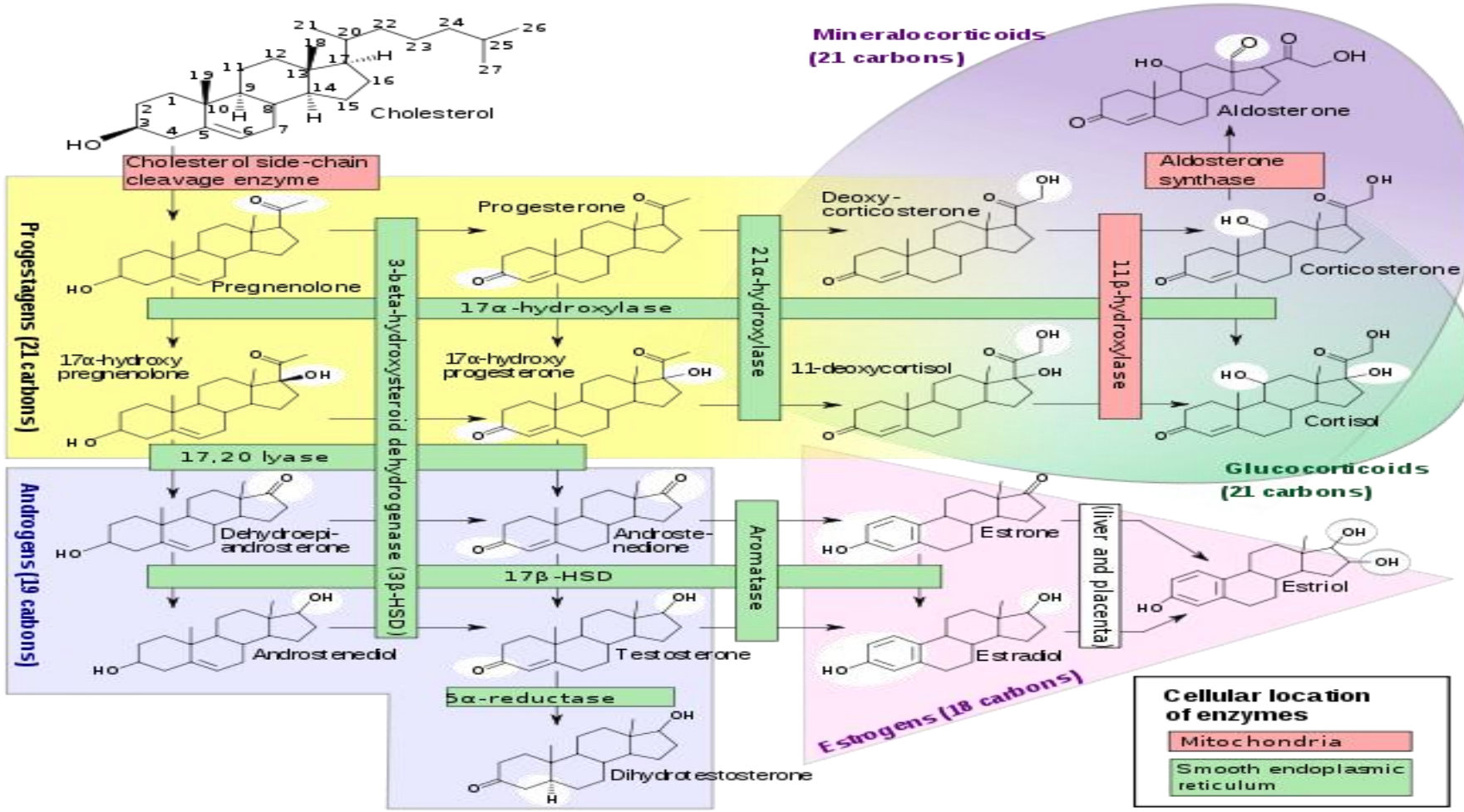

Figure2. 2 Steroidogenesis (Adapted from Krone et al., 2010)

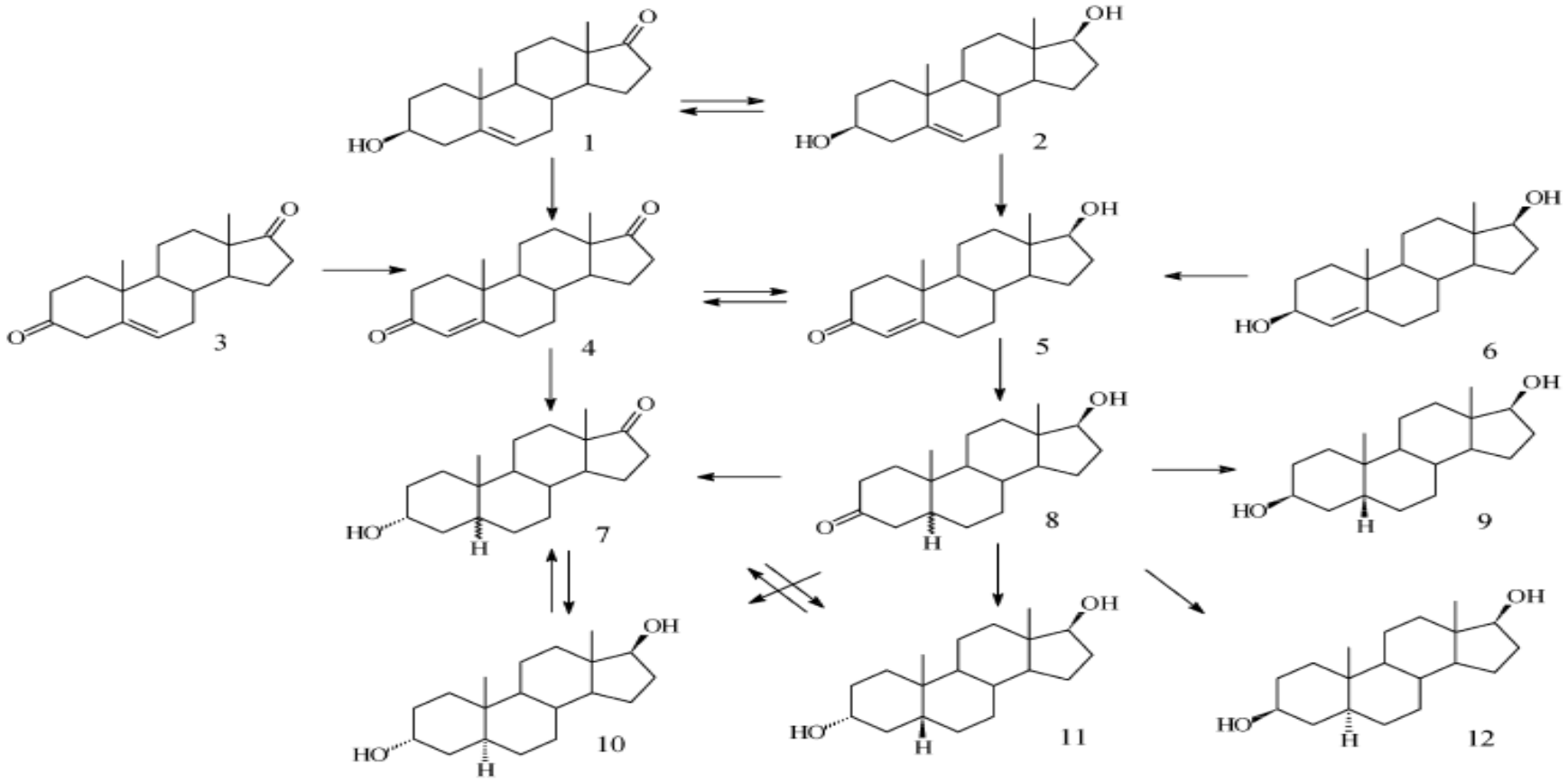

Figure 2. 3 Metabolic scheme of testosterone and its prohormones: 1, dehydroepiandrosterone; 2, androst-5-ene-3β,17β-diol; 3, androst-5-ene-3,17-dione; 4,androst-4-ene-3,17-dione; 5, testosterone; 6, androst-4-ene-3β,17 β -diol; 7, 5α- and 5β -androstandione; 8, 5αand 5β -dihydrotestosterone; 9, 5β -androstan-3β,17β -diol; 10, androsterone (5α)and etiocholanolone (5 β); 11, 5α - and 5β -androstan-3α,17β -diol; 12, 5α -androstan-3βα,17β –diol (adapted from: Eenoo & Delbeke, 2006)

2.2 Detection of Anabolic Androgenic Steroids in Human Urine in Doping Control

2.2.1 History of Testing

The use of anabolic androgenic steroids by athletes for the purpose of enhancing their performance was first reported in 1950s (Kam & Yarrow, 2005). After the Munich Olympics in 1972, widespread abuse of steroids in sports was realized. Such a misuse has led to the addition of anabolic androgenic steroids to the list of banned substances by the International Olympic Committee. Its Initial tests relied on radioimmunoassay (RIA). RIAs were developed for detecting three steroid groups: 17-methylated steroids, 17-ethylated steroids and nandrolone. Large scale detection of anabolic androgenic steroids abuse was first executed in the Montreal Olympics in 1976 using RIA for screening and GC/MS with magnetic sector analyzer for confirmation purposes. Insufficient cross-reactivity towards different AAS and their metabolites and the high non-specificity have limited the usefulness of RIA, although it was used only as a screening test. In early 1980s, rapid improvements in mass spectrometers allowed doping control laboratories to develop specific, sensitive and comprehensive screening and confirmation methods for the detection of steroid abuse (Bowers et al., 2009).

2.2.2 Strategy of Testing

In sport drug testing, AAS are presently analyzed only from urine samples. In general, the measurements are qualitative in nature; however the detection of the natural steroids abuse those are structurally identical to those produced endogenously

like testosterone; requires quantification. All methods used are based on chromatography combined with mass spectrometry. Depending on the steroids, the required performance limit is either 2 or 10 ng/mL. Currently, laboratories routinely analyze samples only for the presence of free and glucuronide-conjugated steroids (WADA, TD2009MRPL).

The analysis of AAS as well as of other banned substances is divided into two categories: the screening procedures and the methods of confirmatory analysis. The aim of screening is to put suspicious samples under further analysis. An ideal screening method should be simple, fast, selective, sensitive and not prone to wastage of sample unnecessarily. Because of the large number of banned substances, multi-analyte screening methods are favored in order to keep the number of separate procedures at the minimum (Kicman & Gower, 2003)

All presumptive positive samples found by screening are confirmed by reanalyzing them along with a drug-free sample and with appropriate reference samples using highly specific and sensitive analytical methods. Drugs are verified by their chromatographic retention times and by mass spectral properties compared to certified reference compounds. Confirmation measurements are recommended to be performed in full scan mode; however, the confirmation of low concentrations is permissible using monitoring of selected ions. The criteria of identification are strictly specified in order to harmonize the practice and to ensure defensible results (Hatton & Catlin, 1987)

2.2.3Analytical Techniques for Detecting Steroids

The basic method to detect AAS was developed by Donike et al. Their analyses were performed on bench-top quadrupole MS coupled with capillary GC.

Samples were cleaned up using XAD-2 solid phase material, followed by hydrolysis of conjugated metabolites using β-glucuronidase enzyme and liquid-liquid extraction (LLE) with diethyl ether at alkaline pH (Shackleton, 2009). Prior to GC/MS in selected ion monitoring mode (SIM), steroids were derivatized with a mixture of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), trimethyliodosilane and antioxidant to convert steroid hydroxyl and keto groups to their unique trimethylsilyl(TMS) ethers and enol ethers (Bowden et al., 2009). Modifications from the original method include for example solid phase extraction (SPE) with C18 cartridges, LLE with tertbutyl methyl ether and a combination of pretreatment and finally an analysis of free and conjugated steroids. The latest variation includes direct hydrolysis of urine, followed by LLE and derivatzation using deuterated androsterone glucuronide and etiocholanolone for quality assurance. A typical GC/MS-SIM run takes 20-30 min and incorporates 10-15 time to program the acquisition groups of 15-20 ions. Depending on the steroid, the limit of detection (LOD) between 2-30 ng/mL can be achieved. Negative and positive ion chemical ionization has been applied to different derivatives of many AAS metabolites but without any significant enhancement in terms of sensitivity. Since sufficient LOD are not achieved for all steroids with the basic method, laboratories have been obliged to search for complementary analytical methods to screen and confirm steroids (Shackleton, 2009).

The development of GC/MS showed steady progress over the next 40 years; a matter that results in the inexpensive and robust bench-tops that are used today. In doping control, full steroid profile was fundamentally introduced as it undoubtedly could form a part of ‘Athletes Biological Passport’ that is currently being proposed for all athletes (Shackleton, 2009). Athletes Biological Passport is a collection of

carefully selected individual information which will assist Anti-Doping Organizations in differentiating between deviations of specific markers that may be naturally occurring from those deviations that are likely caused by doping (Sottas et al., 2010).

2.2.3.1 Measurement of Steroids by Chromatography and Mass Spectrometry

Due to significant advances in mass spectrometry (MS) technology, the routine analyses of steroid hormones are facilitated in clinical and research laboratories. Nowadays, clinical laboratories can achieve greater throughput of patient samples with high accuracy and precision. In addition to that, the MS methodology is now sufficiently rapid and robust for quantitating steroid hormones.

In the past decades, high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (LC/MS/MS) has a significant development on steroid hormones analysis. Besides that, the development of an electrospray source by Nobel Laureate John B. Fenn, PhD., 1990, and the subsequent development of atmospheric chemical ionization have facilitated routine analysis of steroids in clinical laboratories (Fenn et al. 2005). Such a technological progress has facilitated the ionization of the analytes present in liquid droplets and sprayed the molecules directly into the mass spectrometer from the HPLC. This advancement has further allowed having a simple coupling of the liquid chromatography (LC) eluent, which reduces the complexity of the assay and shortens the assay run time dramatically (Stanczyk & Clarke, 2010).Most of the liquid chromatography mass spectrometry (LC/MS) based methods for the detection of anabolic steroids usedtandem mass instruments in selected reaction monitoring (SRM) mode due to its specificity and selectivity. Additionally, the selection of a specific SRM transition

can minimize the endogenous urinary interferences. Using this approach, free or conjugated steroids could be detected in different doping relevant matrices such as human and horse urine, dietary supplements, plasma or hair. The main limitation of this approach (common to all LC/MS approaches) is the poor ionization efficiency of steroids in electrospray ionization; this is especially so for steroids that do not have a conjugated keto moiety (Pozo et al., 2011). In addition, LC typically does not provide resolution as high as that of GC and can have difficulty in separating analytes with a very closely related structure (Stanczyk & Clarke, 2010).

While the advent of LC-MS/MS in the past decades has resulted in dramatic improvements in the sensitivity, specificity and automation of serum steroid hormone measurements, there are still situations where a GC/MS or GC/MS/MS assay provides higher chromatographic resolution and even sensitivity. A particular strength of GC-MS and GC-MS/MS is their high applicability to measure large numbers of structurally similar analytes. Accordingly, they have remained the most powerful discovery tool for defining steroid disorder metabolites.

2.2.3.1.1 Mass Spectrometry

The mass spectrometer consists of an ion-source, a mass analyser, a detector and a data system (Figure 2.4). Sample molecules are admitted to the ion-source where they are vaporised and ionised. The ions are then separated according to their mass-to-charge ratio (*m/z*) in the mass analyser and are finally detected. The resulting signals are transmitted to the data system and a plot of ion-abundance against *m/z* corresponds to a mass spectrum. In many cases, a separating inlet device precedes the ion-source, so that complex mixtures can be separated prior to admission to the mass spectrometer. Today, the separating inlet device is usually either a capillary

gaschromatographic (GC) column or a high performance liquid chromatographic (HPLC) column, although the capillary electrophoresis or thin layer chromatography can also be interfaced with mass spectrometry (Watson & Sparkman, 2007)

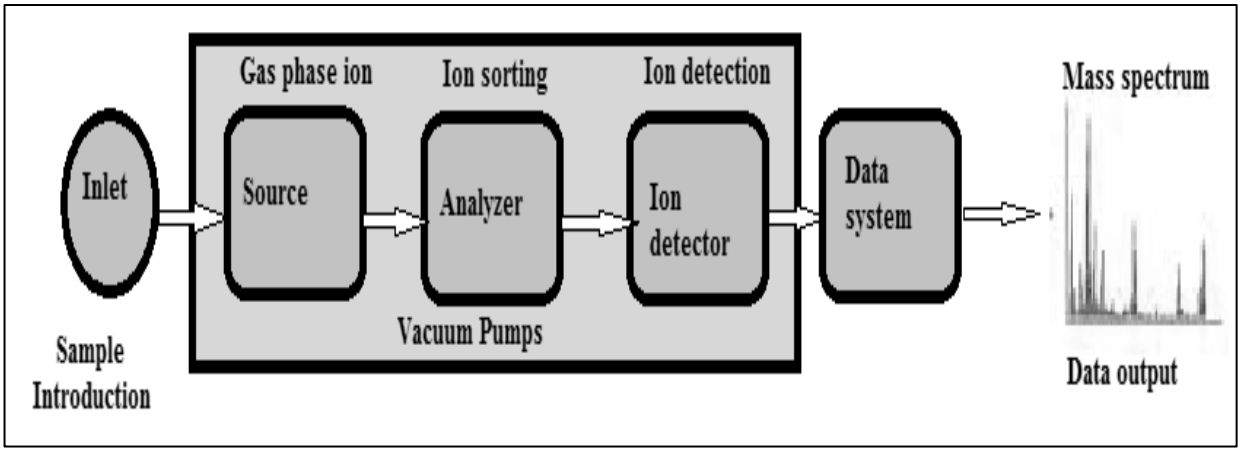

Figure 2. 4 Schematic diagram for Mass Spectrometer

Mass spectrometry technique was used for the steroid metabolism by the late 1930. Currently, the most common type of MS analyzer in doping analysis is the single quadrupole MS system, which can be used either in the full scan mode or in selected ion or selected ion monitoring (SIM) mode in screening protocols. GC was the first analytical techniques used to detect prohibited substances in athletes like anabolic agent and GC/MS has become the workhouse of doping control laboratories worldwide. Screening of these substances is performed by either using the full scan mode or SIM mode with single quadrupole mass analyzer. SIM can also be used for confirmation of a suspect positive by determining the relative ratios of the multiple ions being monitored for each target compound and comparing these to the ratios obtained for authentic standards (Latiff & Churley, 2009).

A GC/MS system in full scan mode will monitor a range of masses known as mass to charge ratio. A typical mass scan range will cover from 35-500 m/z four

times per second and will detect compound fragments within that range over a set period of time. Laboratories have extensive computer libraries that contain mass-spectra of many different compounds that can be compared to the unknown analyte spectrum. The full scan mode is very useful when identifying unknown compounds in a sample and providing confirmation of results from GC using other types of detectors.

Operating GC/MS in SIM mode allows for the detection of specific analytes with increased sensitivity relative to full scan mode. In SIM mode, the MS gathers data for masses of interest rather than looks for all masses over a wide range. Because the instrument is designed to look for only masses of interest, it can be specific for a particular analyte of interest. Typically, two to four ions are monitored per compound and the ratios of those ions will be unique to the analyte of interest. In order to increase sensitivity, the mass scan rate and dwell time, which is the time spent in looking at each mass should be adjusted.

2.2.3.1.2 Gas Chromatography/Tandem Mass Spectrometry

Enhancement in sensitivity and selectivity can also be obtained using MS/MS by monitoring selected product ions that formed in collision-induced dissociation of a precursor ion (Watson & Sparkman, 2007). Most steroids could be analyzed at concentrations lower than the required performance limits of 2 and 10 ng/mL (WADA, TD2010 MRPL). Improvements in the LOD along with a better selectivity, helped shorten the overall analysis time significantly. Van Eenoo et al. (2011) have described a GC/MS/MS method for detecting a wide range of different doping agents. AAS or their metabolites could be analyzed within 8 minutes. MS/MS measurements of AAS have been mostly carried out with quadrupole ion trap

instruments. In addition to screening purposes, MS/MS has been used in confirmatory analysis.

2.2.3.1.2.1 Principle of Gas Chromatography/Tandem Mass Spectrometry (GC/MS/MS)

When a second phase of mass fragmentation is added; for example using a second quadrupole in a quadrupole instrument, it is called tandem MS (MS/MS). MS/MS can sometimes be used to quantitate low levels of target compounds in the presence of a high sample matrix background.

The first quadrupole (MS1) is connected to a collision cell (MS2) and another quadrupole (MS3) (Figure 2.5). Both quadrupoles can be used in scanning or static mode, depending on the type of MS/MS analysis being performed. The types of analysis include product ion scan, precursor ion scan, Selected Reaction Monitoring (SRM) (sometimes referred to as Multiple Reaction Monitoring (MRM)) and Neutral Loss Scan. For example, when MS1 is in static mode (looking at one mass only as in SIM), and MS3 is in the scanning mode, the product ion spectrum which is also called daughter spectrum can be obtained. From this spectrum, a prominent product ion is selected which can be the product ion for the chosen precursor ion. The pair is called a transition that forms the basis for SRM. The latter is characterized by being highly specific and by its ability to eliminate the matrix background virtually.

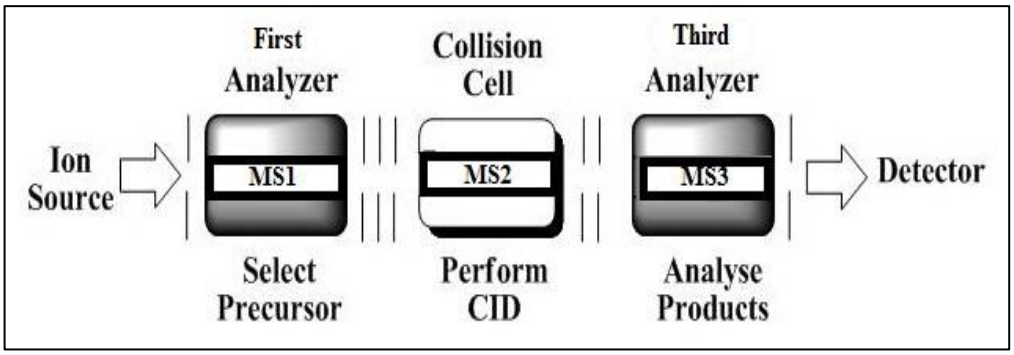

Figure 2. 5 Schematic diagram of MS/MS adapted from: http://www.medandlife.ro/medandlife418.html. Retrieved 28/9/2011

There are four main possible scan experiments that can be conducted by using MS/MS. Product ion scan in which the first MS is set to select an ion of a known mass, will be fragmented in the second MS. The third MS is then set to scan the entire m/z range, giving information on the fragments produced. From the ion fragmentation information, the structure of the original ion can be deduced. This experiment is commonly performed to identify the transitions used for the quantification by tandem MS. When using the precursor ion scan, a certain product ion is selected in third MS, and the precursor masses are scanned in the first MS. In Neutral loss scan mode, both the first MS and the third MS are scanned together, but with a constant mass offset. This allows the selective recognition of all ions which have been fragmentated in the second MS; a matter that leads to the loss of a given neutral fragment (e.g., H_2O, NH_3). Similar to the precursor ion scan, this technique is also useful in the selective identification of closely related class of compounds in a mixture. The fourth type is the selected reaction monitoring (SRM)/ Multiple reaction monitoring (MRM). Both the first and third MS are set to a selected mass, allowing only a distinct fragment ion from a certain precursor ion to be detected. This technique is a very selective analysis mode, which can result in increased sensitivity (Watson & Sparkman, 2007).

2.2.3.1.2.2 Advantages and Application of GC/MS/MS:

The triple quadrupole MS provides superior sensitivity and selectivity for the compound analysis in a complex matrix in comparison to the single quadrupole MS. This is because of the specificity of tandem MS. Moreover, one of the important advantages is the elimination of background interferences that allows a very low detection limits for quantification like femtograms. Improvements in MS sensitivity are achieved through increasing the signal strength and reducing the chemical noise.

The GC/MS/MS instrument is very robust in high throughput application areas which include pesticides and herbicides that can be encountered in foods especially food those that have very complex volatile backgrounds. The GC/MS/MS has been shown to analyse tetrahydrocannabinol (THC) derivatives at low detection limits. Besides, it also shows great promise in the analysis of human fluids in case of intaking drugs and drugs of abuse.

The strength of tandem MS is the one that does not need to know the structure of an analyte to detect it. Rather, some very specialized operational modes can be used to identify compounds that have chemical resemblances to other well understood compounds in a homologous series of compounds. An example of this can be found during the process of identifying metabolites of new therapeutic entities after being administrated to laboratory animals or human (Clarke et al., 2001).

A Chinese study investigated the physiological concentration of anabolic steroids in human hair of Chinese subjects by using GC/MS/MS. The measurement of anabolic steroids in hair is necessary in order to distinguish between the pharmaceutical steroids and natural steroids (Shen et al., 2009).

Another GC/MS/MS method was developed and applied to the analysis of Chinese cooked foods. The results demonstrated the potentiality of the GC/MS/MS method in analyzing trace food-derived hazardous compounds in a complex food matrix such as meat samples (Zhang et al., 2008).

In addition, another scientist showed that GC/MS/MS can be used for determining of pesticide residues in crops and dry animal feed. This is because it gives a much higher degree of certainty in analyte identification than in any single stage mass spectrometry technique. Such ability can be attributed to the fact that because isobaric interference are avoided and multiple-component spectra can be resolved. Thus, the confirmation of target analytes can be achieved with higher levels of confidence (Stanis, 2007).

2.3 *Eurycoma Longifolia* Jack (Tongkat Ali)

Eurycoma longifolia Jack is an herbal plant native to South East Asian countries, mainly Malaysia and Indonesia. It is popularly recognized as 'Tongkat Ali', which means the 'walking stick'; that is derived from the presence of long twisted roots (consider Figure 2.6) (Bhat & Karim, 2010).

Almost all parts of the *Eurycoma longifolia* plant have traditionally been used for therapeutic purposes. The plant extract have also been traditionally used due to its antimalarial, aphrodisiac and antipyretic activities (Kuo et al., 2003). The roots are the most valuable component and are used for the treatment of aches, persistent fever, malaria, sexual insufficiency, dysentery, glandular swelling, and as health supplements (Bhat & Karim, 2010).

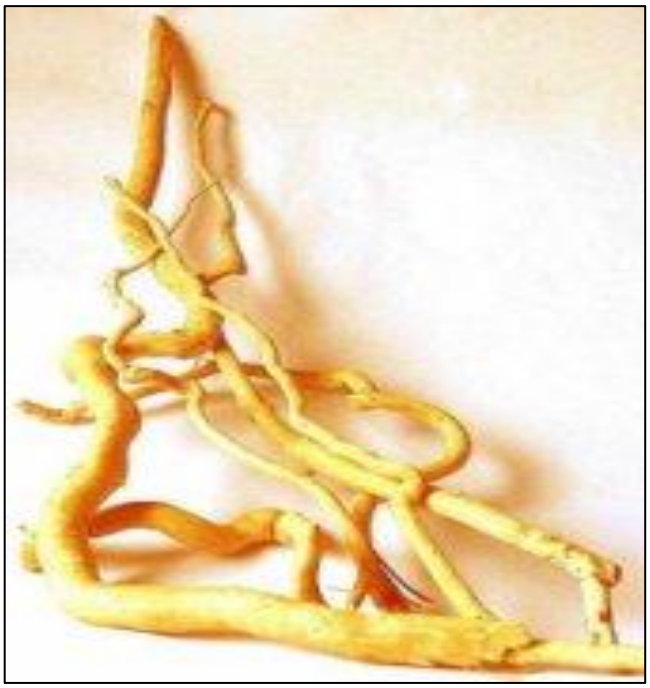

Figure 2. 6 *Eurycoma longifolia* root (adapted from http://tong-kat-ali.com/buying-tongkat-ali-extract.html) Retrieved 5/9/2011

As mentioned before, the root extract of *Eurycoma longifolia* has gained wide recognition due to its unique feature of enhancing the virility and sexual competency, when the prepared extract is consumed. Besides, this plant has traditionally gained wide popularity for its aphrodisiac activities, it is necessary to validate the product for consumer safety and to declare that it safe for human consumption. Though several extensive researches are currently being undertaken worldwide, there is still a wide gap in the scientific information generated with reference to the human model system (Bhat& Karim, 2010).

There has been a remarkable increase in the demand for the products of this plant. Moreover, nearly 200 of *Eurycoma longifolia* products highlighted the aphrodisiac properties, available in the health food market (Zanoli et al., 2009). *Eurycoma longifolia* products are available either in the form of raw crude powder of roots, as in the case of capsules mixed with other aphrodisiac herbs or as an additive

mixed with coffee and ginseng or in certain health products as a replacement for ginseng (Low & Tan, 2007). The regular intake of the root extracts is believed to enhance the testosterone levels. An increased interest has also been created among the athletes and individuals involved in body building with respect to using such an extract to enhance the muscle mass, strength and the overall performance (Bhat& Karim, 2010).

2.3.1 Reviews on the biological effects of *Eurycoma longifolia*

A review study on the ethanobotany and pharmacological importance of *Eurycoma longifolia* was carried out in 2010. Such study has stated that: "The plant parts have been traditionally used for its antimalarial, anti-diabetic, antimicrobial and anti-pyretic activities" (Bhat & Karim, 2010). Furthermore, there are many other important medical areas of interest in *Eurycoma longifolia*as stated in the previous study. Most of the Southeast Asians consume it due to its positive impact on health. In 2001, Malaysian scientific researchers opened their peer-reviewed, Medline-archived report on the effect of *Eurycoma longifolia* on laboratory rats. The study maintained "that *Eurycoma longifolia*commonly known as Tongkat Ali has gained notoriety as a symbol of man's ego and strength by the Malaysian men" (David, 2005).

Another group of scientists confirmed that *Eurycoma longifolia*has the capacity to reverse the inhibitory effects of oestrogen on testosterone (Nazrun et al., 2010). Another study was conducted and found that the root of *Eurycoma longifolia* improved the sexual performance of male rats after acute administration. The authors suggest that the effect could be mainly ascribed to increasing the levels of testosterone in serum (Zanoli et al., 2009). In another experiment conducted on male

rats, it was reported that the extract of *Eurycoma longifolia* increased significantly the levels of plasma testosterone of treated rats in comparison to that of the control and infertile animals (Wahab et al., 2010).

After many scientific publications on the effects of *Eurycoma longifolia,* researchers in the field of sports medicine began to look into the anabolic potential of this plant. In a placebo-controlled human study with healthy young men in a weight training program, it was found that "the body mass of the treatment group showed a significant increment, from 52.26 kg to 54.39 kg ($p=0.012$)". Furthermore, "the increased in strength in the treatment group was larger than in the placebo group (6.78% and 2.77% respectively)''. The mean arm circumference of the treatment group increased significantly by 1.8 cm after the supplementation. However, there was no significant increase in the placebo group. The results suggested that the water soluble extract of *Eurycoma longifolia* increased fat free mass, reduced body fat, and increased muscle strength and size (Hamzah & Yusof, 2003).

The anabolic impact of *Eurycoma longifolia* has been also confirmed in the animal model, when the size and weight of just one muscle were measured in treated and untreated rats of equal size. The results showed that 800 mg/kg of butanol, methanol, water and chloroform fractions of *Eurycoma longifolia* have significantly increased ($p<0.05$) the levator ani muscle of the rats (Ang & Ngai, 2001).

Due to these significant discoveries, a growing number of athletes and body-builders are now using the extract of *Eurycoma longifolia*as an androgen to improve muscle size, strength, and enhance sport performance instead of using dangerous and potentially lethal steroids.

Apart from the anabolic and testosterone-related effects of *Eurycoma longifolia,* the antimalarial (Kuo et al., 2004), antibacterial (Kuo et al., 2003), antipyretic, antiulcer, antitumor (Miyake et al., 2010), and cytotoxic properties have been well documented. Taiwanese scientists isolated 65 biochemical compounds from the roots of *Eurycoma longifolia.* Out of this number of compounds, only ten exhibited strong cytotoxicity towards human lung and breast cancer cell lines (Miyake et al., 2010). Another group of researchers discovered several new biochemical compounds in *Eurycoma longifolia* and screened them for cytotoxic properties. They concluded that different fractions were effective against different types of cancers (Tee et al., 2005). In another study, the authors shared that fractions of *Eurycoma longifolia* extract induced apoptosis in cancer cells (Jackson et al., 2010).